MOLECULAR ANALYSES OF ENDOCRINE AND NUTRITIONAL FACTORS THAT AFFECT DIVISION OF LABOR AND HEALTH IN HONEY BEES (*APIS MELLIFERA*)

BY

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DISSERTATION

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ABSTRACT

In animals, food-related behaviors are linked to health, reproduction and survival. As such, the processes that regulate these behaviors involve complex interactions between the environment and an individual's physiological state. In social animals, social interactions additionally influence food choices, and these choices are regulated internally through crosstalk between the brain and the body. Here, I employ a transcriptomic approach to understand how environmental and physiological factors mediate the onset of foraging behavior in the highly social honey bee (*Apis mellifera*). In Chapter 1, I provide a detailed overview of each experimental chapter included in this dissertation. In Chapter 2, I investigate the molecular mechanisms by which a peripheral storage protein, vitellogenin, regulates foraging onset. Using RNA interference and gene expression profiling, I show that vitellogenin elicits a strong transcriptomic response in the brain that closely resembles the response elicited by another key regulator of foraging onset, juvenile hormone. These results suggest these two physiological factors act through common pathways to regulate honey bee behavior. To provide greater mechanistic insights, in Chapter 3, I investigate a brain region, the *pars intercerebralis,* which is known to interact with nutritional cues and with juvenile hormone in other insect species. To investigate whether the *pars intercerebralis* is involved in regulating foraging behavior in honey bees, I specifically tested whether this brain region is responsive to dietary manipulations and alterations in juvenile hormone levels. The results of this study suggest the *pars intercerebralis* is implicated in the onset of foraging behavior and provide evidence for changes in *pars intercerebralis* function related to honey bee social evolution. Lastly, in Chapter 4, I explore a different aspect of the relationship between nutrition and honey bees. I examine the effects of apicultural food supplements on gene expression in adipose tissue. These results show that honey elicits a distinct

transcriptomic response, indicating honey provides nutritional components that are absent in commonly used apicultural food supplements.

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TABLE OF CONTENTS

CHAPTER 1:
GENERAL INTRODUCTION

Food-related behaviors are core elements linked to health, reproduction and survival. As such, the processes that govern food-related behaviors are intricately regulated and involve complex interactions between an individual's physiological state and the environment (DiLeone et al., 2012; Morton et al., 2006). In social animals, food availability and social experiences dictate food choices (Galef and Giraldeau, 2001), and internally these choices are regulated by two-way interactions between the brain and the body (Morton et al., 2006). A major focus of my dissertation research has been to use transcriptomics to understand how environmental and physiological factors mediate the onset of foraging in the highly social honey bee (*Apis mellifera*). I also studied the effects of apicultural food supplements on gene expression in adipose tissue in order to investigate the impact of these supplements on honey bee physiology and health.

Honey bee societies are extremely complex and are known for dividing tasks among colony members. The vast majority of the bees in a colony are workers, which are essentially sterile females that perform all of the non-reproductive tasks related to colony growth and maintenance. Workers divide colony tasks using an age-related system that is based on a process of individual behavioral maturation (Winston, 1987). Over the past 20 years, behavioral maturation has been studied extensively and used to understand how environmental and physiological factors regulate individual behavior and shape insect societies (Smith et al., 2008; Toth and Robinson, 2007).

Workers spend their first 1-3 weeks of adult life inside the hive performing tasks such as brood care (nursing behavior) and food storage; they then switch to activities outside the hive for their

remaining 1-2 weeks of life, performing tasks such as nest defense and foraging for nectar and pollen (Winston, 1987). Importantly, the pace of behavioral maturation is not rigid, because it must remain responsive to colony needs. Individual bees are sensitive to changes in colony food stores and shifts in colony demography. Precocious foraging, for example, can be induced experimentally by a sudden depletion in colony food stores (Schulz et al., 1998), as well as by a depletion of a colony's foraging force (Huang and Robinson, 1992).

Furthermore, the shift from hive work to fieldwork requires a major physiological transformation, which involves changes in exocrine gland development, flight metabolism, endocrine signaling and gene expression in several different tissues, including the brain (Robinson, 2002). Dietary changes and changes in nutritional physiology also occur: young bees feed on pollen and have high abdominal lipid levels, while older bees mostly feed on honey and nectar and have low levels of abdominal lipids (Crailsheim et al., 1992; Toth and Robinson, 2005). Some of these changes are correlates of behavioral maturation, enabling bees to become specialists for particular tasks. The development of different glands, for example, enables bees to perform nursing behavior or to be efficient foragers (Huang et al., 1994), whereas other changes are regulatory changes that are able to shift bees from hive work to foraging.

Behavioral maturation in honey bees is regulated by changes in gene expression, endocrine signaling and nutritional physiology. Thousands of changes in brain gene expression are associated with behavioral maturation (Alaux et al., 2009; Whitfield et al., 2003) and a subset of these are thought to integrate environmental cues and converge on hormonal signals downstream. Among hormones, juvenile hormone (JH), a pleiotropic insect hormone, plays a

prominent role in worker division of labor. JH levels increase during behavioral maturation (Huang and Robinson, 1995; Huang et al., 1991; Rutz et al., 1976) and several manipulative experiments, including removal of the neuroendocrine glands that produce JH, have demonstrated that elevated JH increases a bee's likelihood to become a forager (Robinson, 1987; 1985; Sullivan et al., 2000). JH elicits brain gene expression changes that parallel differences in expression between nursing and foraging bees (Whitfield et al., 2006). In addition, JH is known to have complex interactions with peripheral nutritional signals (Corona et al., 2007; Guidugli et al., 2005).

In many insect species, JH acts as a gonadotropic hormone, by directly stimulating the synthesis of vitellogenin (Vg) from fat body tissue (Wyatt and Davey, 1996). However, in adult worker honey bees, in which ovaries are undeveloped, JH and Vg have an antagonistic relationship: treatment with a JH analog causes a reduction in Vg (Corona et al., 2007) and a decrease in Vg causes an increase in JH levels (Guidugli et al., 2005). Moreover, experimental knockdown of Vg induces precocious foraging behavior (Nelson et al., 2007). Early work proposed that this novel relationship between JH and Vg, and more generally the loss of JH as a gonadotropic signal, enabled JH to play a central role in mediating worker division of labor (Robinson and Vargo, 1997). The work presented in Chapters 2 and 3 of this dissertation builds on these findings and ideas by using transcriptomics to provide mechanistic insights into how JH and Vg elicit changes in behavior.

In Chapter 2, I tested the hypothesis that Vg induces precocious foraging through changes in brain gene expression. As in other insects, Vg in honey bees is produced in the fat body and is

transported into cells via receptor-mediated endocytosis. Thus far, there is no evidence that Vg or its receptor are expressed in the brain (Guidugli-Lazzarini et al., 2008), but since Vg elicits changes in behavior a reasonable assumption is that Vg has indirect effects on brain gene expression. In Chapter 2, I knocked down the expression of the *vg* gene in the abdomen with RNA interference and examined effects on brain gene expression with microarrays. I found Vg strongly influenced brain gene expression in honey bee workers and many of the same genes show maturation-related differences, but the direction of change for the genes in these two contrasts was not correlated. By contrast, *vg* knockdown targeted many of the same genes that are regulated by JH and there was a significant correlation between these two contrasts, suggesting Vg may act through JH to regulate behavioral maturation.

In Chapter 3, I used laser capture microdissection and gene expression profiling to explore whether the *pars intercerebralis* (*PI*), a neurosecretory region of the brain, might be involved in behavioral maturation. Axons from the *PI* extend back to the neuroendocrine glands that produce JH (Eichmüller et al., 1991). Moreover, there is evidence of co-regulatory interactions between nutrition-related neuropeptides and JH (Tatar et al., 2001). I explored whether the *PI* is involved in mediating nutritional and endocrine effects on behavioral maturation by first investigating transcriptomic differences in the *PI* of nurses and foragers to quantify baseline differences. I then tested the effects of dietary alterations and JH analog treatments on *PI* gene expression. I found that there are thousands of gene expression differences between the nurse and forager behavioral states in the *PI* and that many of these differences relate to changes in protein turnover, energy metabolism and neuropeptide signaling. I also found that JH analog treatments caused forager-like changes in *PI* gene expression, indicating the presence of a JH feed-forward mechanism in

honey bee foragers. By contrast, diet manipulations elicited few gene expression changes in the *PI* and the majority of these changes were not consistent with behavioral maturation. These results indicate that gene expression in *PI* is responsive to behavioral maturation and a subset of the changes in expression likely mediate foraging onset through co-regulatory interactions with JH.

Lastly, in Chapter 4, I examined another aspect of the relationship between nutrition and honey bees. My goal was to study the effects of apicultural carbohydrate supplements on fat body gene expression to understand their impact on honey bee physiology and health. Honey bees are routinely fed high fructose corn syrup or sucrose after the harvesting of honey or during periods of nectar dearth and there is increasing concern that nutrition and other extrinsic factors are weakening bees and contributing to the "Colony Collapse Disorder" associated with the massive losses in honey bee colonies over the past 8 years. In laboratory trials, I fed bees high fructose corn syrup, sucrose, or honey and evaluated the effects of these diets on fat body gene expression. The fat body is a peripheral nutrient-sensing organ analogous to vertebrate liver and adipose tissue (Arrese and Soulages, 2010); therefore, I was directly investigating the effects of these diets on nutrient storage, energy metabolism and immune function. I found that honey elicits a transcriptional profile that is distinct from the two carbohydrate supplements. The expression differences included genes involved in protein metabolism and oxidation-reduction, including some involved in tyrosine metabolism and xenobiotic detoxification. My results suggest bees receive nutritional components from honey that are absent from sucrose and high fructose corn syrup.

Together, the studies presented in this dissertation provide mechanistic insights into the relationships between nutrition, physiology and behavior in honey bees. My results include straightforward connections linking honey – a bee's natural carbohydrate source – to nutritional physiology and health. They also reflect the complexity with which behavioral maturation is regulated. In this regard, my results suggest that JH has not only lost its traditional role in reproduction but also has become disassociated from its traditional connection to nutritional inputs. Although all of my work is based on measurements of an individual's transcriptomic state, the transcriptomic state of individuals is inextricably linked to health and function at the colony level in this highly social species.

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CHAPTER 2:
BRAIN GENE EXPRESSION CHANGES ELICITED BY PERIPHERAL *VITELLOGENIN* KNOCKDOWN IN THE HONEY BEE[1]

ABSTRACT

Vitellogenin (Vg) is best known as a yolk protein precursor. Vg also functions to regulate behavioral maturation in adult honey bee workers, but the underlying molecular mechanisms by which it exerts this novel effect are largely unknown. We used abdominal *vitellogenin* (*vg*) knockdown with RNAi and brain transcriptomic profiling to gain insights into how Vg influences honey bee behavioral maturation. We found that *vg* knockdown caused extensive gene expression changes in the bee brain, with much of this transcriptional response involving changes in central biological functions such as energy metabolism. *vg* knockdown targeted many of the same genes that show natural, maturation-related differences, but the direction of change for the genes in these two contrasts was not correlated. By contrast, *vg* knockdown targeted many of the same genes that are regulated by juvenile hormone (JH) and there was a significant correlation for the direction of change for the genes in these two contrasts. These results indicate that the tight co-regulatory relationship that exists between JH and Vg in the regulation of honey bee behavioral maturation is manifest at the genomic level and suggest that these two physiological factors act through common pathways to regulate brain gene expression and behavior.

[1] Wheeler, M.M., Ament, S.A., Rodriguez-Zas, S.L., Robinson, G.E. (2013) Brain gene expression changes elicited by peripheral *vitellogenin* knockdown in the honey bee. *Insect Mol Biol* **22**(5): 562-73.

INTRODUCTION

Honey bees are well-known for their division of labor among workers. This division of labor enables honey bee colonies to perform tasks simultaneously and efficiently and is a key component of their ecological success (Oster & Wilson 1978). In honey bees, worker-worker division of labor is based on a process of behavioral maturation: for the first 2-3 weeks of adult life, workers perform tasks inside the hive, such as nursing (brood care) and food storage, and as they become older, they progress to tasks outside, including foraging for pollen and nectar (Winston 1987). In recent decades, this hive-to-field behavioral maturation has become a model system to understand the environmental, physiological and molecular factors that shape division of labor in insect societies (Toth & Robinson 2007; Smith *et al.,* 2008).

Numerous studies have shown that the process of behavioral maturation is complex and is regulated by a variety of intrinsic and extrinsic factors (Chandrasekaran *et al.,* 2011; Ament *et al.,* 2012a). Among these, changes in diet and nutritional physiology are known to regulate behavioral maturation (Schulz *et al.,* 1998; Toth *et al.,* 2005; Ament *et al.,* 2008). The most prominent physiological factors include juvenile hormone (JH) and vitellogenin (Vg). JH titers increase during behavioral maturation and high JH titers are generally associated with foraging behavior (Rutz *et al.,* 1976; Fluri *et al.,* 1982; Huang & Robinson 1995). Treatment with JH analogs (JHA) induces precocious foraging behavior (Robinson 1985; Robinson 1987) while removal of the JH-producing glands delay it, and this deficit can be rescued with hormone replacement treatment (Sullivan *et al.,* 2000). RNAi knockdown of *usp*, a transcription factor involved with JH signaling, also causes a delay in behavioral maturation (Ament *et al.,* 2012b).

In addition, Vg, a fat body-produced storage protein, is involved in a co-regulatory loop with JH (Amdam & Omholt 2003).

Vg is best known as a yolk protein precursor, and nutritional- and JH-mediated induction of Vg synthesis is well established in many insects (Wyatt & Davey 1996). Similarly, in honey bee workers, Vg levels are modulated by ingested nutrients, especially proteins (Bitondi & Simoes 1996). However, unlike most insects, JH and Vg have a mutually repressive relationship in adult worker honey bees; high Vg levels occur in nurse bees and RNAi knockdown of *vg* in the fat body leads to elevated JH (Guidugli *et al.,* 2005) and precocious foraging (Nelson *et al.,* 2007). Regulation of behavioral maturation is a novel function for Vg, but the molecular mechanisms by which this peripherally circulating storage protein affects behavior are not known.

We used brain transcriptomic profiling to gain insights into how Vg might influence honey bee behavioral maturation, taking advantage of prior microarray studies to provide a basis for comparative analysis. Behavioral maturation is associated with large-scale gene expression changes in the brain (Whitfield 2003; Alaux *et al.,* 2009a) and many of these gene expression changes can be attributed to specific factors such as, age and behavior (Whitfield *et al.,* 2006). In addition, diet and JH are known to influence a subset of the nurse-forager gene expression differences (Ament *et al.,* 2012a; Whitfield *et al.,* 2006). We hypothesized that *vg* RNAi causes gene expression changes consistent with diet- and JH-treatments and leads to forager-like changes in brain gene expression. This hypothesis was based on the assumption that Vg also regulates behavioral maturation through transcriptional changes in the brain, in addition to prior

results showing that Vg is nutritionally modulated (Bitondi & Simoes 1996; Nilsen *et al.,* 2011) and *vg* knockdown causes an increase in JH titers (Guidugli *et al.,* 2005).

RESULTS

vg RNAi influences the expression of thousands of genes in the honey bee brain

We performed microarray gene expression profiling of whole brains from four-day-old adult bees treated with *vg* RNAi. We also measured brain gene expression for two age- and genotype-matched control groups: bees injected with saline buffer and bees that were mock manipulated without injection. Bees treated with *vg* RNAi and saline buffer were injected intra-abdominally by Ament *et al.,*(2011). Expression of *vg* in abdominal tissue was reduced by 56-80 % when compared to saline injected bees, and by 65-84% relative to untreated controls (Ament *et al.,* 2011).

Gene expression profiling revealed that Vg influences the expression of thousands of genes in the honey bee brain. A total of 4,726 (37.6%) transcripts were differentially expressed (ANOVA, FDR < 0.05) in the brain. However, *vg,* itself, was expressed at very low levels in the brain (detected in less than 50% of microarrays analyzed) and was not differentially expressed across treatment groups (ANOVA, FDR < 0.05). Post-hoc contrasts indicated that 3,138 of these transcripts were differentially expressed between *vg* RNAi-treated bees and saline-injected controls, while 4,276 transcripts were differentially expressed between the *vg* RNAi treatment group and bees that received no injection. The lists of differentially expressed genes for *vg* RNAi

vs. saline and for *vg* RNAi vs. no injection controls showed a high degree of similarity. A total of 2,796 transcripts overlapped between these gene lists and there was a strong positive correlation for the fold changes of genes that overlapped between these two gene lists (r = 0.911, *P* <0.0001). These results indicate a robust signature of *vg* knockdown relative to either control, but because saline injection did influence gene expression changes relative to bees that received no injection, we performed subsequent analyses using the *vg* RNAi vs. saline injection contrast only (3,138 genes).

We performed a series of analyses to characterize the pathways and individual genes that were robustly and consistently influenced in the brain by *vg* RNAi. We performed Gene Ontology enrichment analyses using DAVID, (Dennis *et al.,* 2003) to obtain functional insights about these genes. The list of genes was enriched for genes related to core metabolic processes, including "translation" (94 genes, *P* = 7.75e-06), "oxidative phosphorylation" (40 genes, *P* = 1.52e-06), and "glycolysis" (11 genes, *P* = 3.45e-04), as well as canonical neuronal functions such as "synaptic transmission" (45 genes, *P* = 0.004). Further analyses indicated that "translation" (56 genes, *P* = 3.14e-06) and "oxidative phosphorylation" (24 genes, *P* = 2.51e-05) were up-regulated in response to RNAi treatment while biological processes related to carbohydrate metabolism such as "glycolysis" (10 genes, *P* =2.42e-04) and "carbohydrate catabolic process" (14 genes, *P* = 0.006) were down-regulated. In addition, genes associated with "synaptic transmission" (40 genes, *P* =2.55e-11) were also down-regulated in response to *vg* knockdown.

To examine whether *vg* knockdown elicited gene expression effects that were consistent from individual to individual, as well as identify "top predictors" of *vg* RNAi treatment, we performed

class prediction analyses using the support vector machine classification algorithm from the CMA package (Slawski *et al.,* 2008). We calculated both the overall accuracy of the group predictions – a measure of the consistency in the effects of *vg* RNAi across individuals – and the "top predictor" genes whose expression differences were most consistent between groups. The group identities of the *vg* RNAi and saline control bees were predicted with 94% accuracy from the expression levels of top 100 predictor genes. Only one bee – a saline control with a low *vg* expression level – was misclassified. This result corresponded to a specificity (true negatives identified) equal to 1 and a sensitivity (true positives identified) of 0.889. These results show there were consistent effects on gene expression due to *vg* RNAi. Among the "top predictors" of *vg* knockdown were *sirt 6 histone deacetylase* (*sirt6)* (GB17426) and *thyroid hormone receptor interactor 4 (TRIP4)* (GB11274). An ordered list of the top 20 predictors is shown in Figure 2.1.

Expression analysis of six genes with qPCR was used to provide technical and biological validation of the microarray results. Samples from a honey bee colony not included in the microarray experiment were used for biological validation. We selected *ecdysone-inducible gene L3 (Impl3)* (GB13882), *osiris 14* (GB19255), *pancreatic lipase-related protein 2 precursor (PLRP2)* (GB30584), *sirt*6 (GB17426), *leucine-rich repeat protein soc-2 homolog* (*Sur-8)* (GB16542) and *TRIP4* (GB11274). These genes were selected on the basis of fold changes and the cross-validation results. Findings consistent with the microarray results were obtained for 4 out of 6 genes; the exceptions were *PLRP2* and *Sur-8* (Figure 2.2). *ecdysone-inducible gene L3* did not show a significant difference but the trend was consistent with the results observed on the microarrays.

Comparisons with brain gene expression changes related to behavioral maturation

Vg influences other processes in honey bees in addition to behavioral maturation, including aging, immunity, and reproduction. To better characterize the subset of *vg* RNAi-induced transcriptomic responses in the brain specifically associated with behavioral maturation, we compared our results to a previous study, that used the same microarray platform, to compare gene expression in the brains of nurse and forager bees (Alaux *et al.,* 2009a). There was significant enrichment of genes differentially expressed due to *vg* RNAi and those that showed expression differences between nurses and foragers (Table 2.1). However, the majority of the genes that overlapped between these two gene lists were not directionally concordant (Table 2.2) and there was no significant correlation for the direction of these gene expression changes (Table 2.3). We obtained similar results when we performed the same correlation analyses using less or more stringent significance thresholds (Tables 2.S2 and 2.S3), indicating this result is not biased by the subset of genes associated with a particular statistical cutoff. These results indicate that Vg and behavioral maturation are associated with many of the same transcriptomic changes, but only a subset of these changes are in a direction consistent with the causal relationship between Vg and behavioral maturation.

We hypothesized that this result might reflect the possibility that *vg* RNAi targeted genes that are more tightly associated with a specific aspect of behavioral maturation, perhaps either age-related or behaviorally-related changes in gene expression, which previously have been shown to be dissociable in honey bees (Whitfield *et al.,* 2003, Alaux *et al.,* 2009a). The usually linked traits of chronological aging and current behavioral status can be unlinked due to the remarkable

plasticity in division of labor exhibited by honey bees and other social insects (Robinson 1992). Comparing our results with previously published age-related or behaviorally-related changes in brain gene expression (Alaux *et al.,* 2009a), we found that the transcriptomic response due to *vg* RNAi was directionally concordant with both, but stronger for the age-related changes (Table 2.2 and 2.3). These results suggest that Vg is most closely associated with the component of behavioral maturation associated with chronological age.

Comparisons with brain gene expression changes related to diet manipulations and juvenile hormone

Changes in blood titers of Vg are associated with dietary changes that occur during the process of behavioral maturation in honey bees: nurse bees feed on a protein- and lipid-rich diet and have high Vg levels, while foragers feed on a carbohydrate-based diet and have low Vg levels (Fluri *et al.,* 1982; Crailsheim *et al.,* 1992). We compared our results with a microarray study that compared bees fed on a protein rich or protein poor diet (Ament *et al.,* 2012a). We found there was significant enrichment for *vg* RNAi- and diet-induced gene expression changes (Table 2.1); however, the majority of the diet-induced gene expression changes and those induced by *vg* RNAi were not directionally concordant (Table 2.2) and were not significantly correlated (Table 2.3). This enrichment and correlation pattern was observed across all pairwise comparisons with diet, including the age and behavior gene lists. This result suggests that the effects of Vg on brain gene expression and behavioral maturation are similar to, but not identical with, the effects on diet.

Based on the well-known link between Vg and JH, we hypothesized that many of the genes influenced by *vg* RNAi might be related to JH signaling. We compared our results with a previous study that used an earlier microarray platform to examine the effect of the JH analog, methoprene (JHA) on brain gene expression (Whitfield *et al.,* 2006). Comparison of *vg* RNAi and JHA transcriptomic responses show that there was significant overlap between these lists (Table 2.1) and there also was a large percentage of genes that showed concordant expression differences (Table 2.2), which resulted in a significant positive correlation across overlapping genes (Table 2.3). We also confirmed that JHA-related gene expression changes overlapped (Table 2.1) and were positively correlated (Table 2.2) with the behavioral maturation changes reported by Alaux *et al.,* (2009a), as was previously shown using data from a different microarray platform for nurses and foragers (Whitfield *et al.,* 2006). In addition, the JHA gene list was positively correlated with age and behavior-related changes (Table 2.3). These results indicate that, although the transcriptomic responses to *vg* RNAi and JHA treatment are very similar, JHA treatment induces effects more similar to the naturally occurring changes associated with behavioral maturation.

To identify genes in the brain that are influenced by Vg and are potentially important to behavioral maturation, we examined genes that overlapped across the gene lists associated with *vg* RNAi, behavioral maturation and JHA treatment. We found that 73 genes overlapped between these three gene lists (Figure 2.S1). A total of 32 genes were directionally concordant across the gene lists associated with *vg* RNAi, behavioral maturation and JHA treatment (Figure 2.3A). Among these were the *glucose transporter 1* and *osiris 14*. In addition, genes associated with behavioral maturation and *vg* RNAi (but not JHA treatment) included genes involved in

chromatin assembly, *insulin receptor precursor*, *juvenile hormone epoxide hydrolase* and the transcription factor *fruitless* (Figure 2.3B).

DISCUSSION

Our principal finding is that peripheral knockdown of Vg elicits an extensive transcriptional response in the brain. This result is consistent with Vg's novel role in honey bees as a regulator of honey bee behavior and physiology. Our findings provide the first insights into the molecular mechanisms in the brain by which Vg mediates honey bee behavioral maturation.

Vg regulates canonical pathways involved in protein turnover and energy metabolism in the brain; specifically, "translation" and "oxidative phosphorylation" were up-regulated in response to *vg* RNAi while carbohydrate metabolism ("glycolysis") was down-regulated. Down-regulation of carbohydrate metabolism is consistent with the biological functions affected by *vg* RNAi in abdominal tissue, indicating Vg affects the same processes in both brain and abdominal tissue (Ament *et al.,* 2011; Ament *et al.,* 2012a). Ament *et al.,* (2012b) tested this directly using pairwise correlations and co-expression analyses for maturation-related microarray experiments, including *vg* RNAi-treated bees. Their results show correlated transcriptional responses across brain and abdominal tissue; a finding which has also been observed between the brain and ovaries (Wang *et al.,* 2012a). These coordinated transcriptional responses are suggestive of hormonally mediated co-regulation and of "crosstalk" between brain and peripheral tissue. In this study, we cannot discern the precise mechanisms by which *vg* influences brain gene expression. However, given that *vg* and its receptor are expressed in the fat body and the ovaries

(Corona *et al.*, 2007; Guidugli-Lazzarini *et al.*, 2008), it is plausible *vg* exerts its effects on the brain through signaling cascades that originate in peripheral tissue, further highlighting the importance of the brain—fat body—ovary axis in the regulation of behavior and physiology in honey bees.

Additional insights from Gene Ontology enrichment analyses suggest that *vg* RNAi elicited changes in energy metabolism that are different from those previously found to be associated with behavioral maturation. Specifically, *vg* RNAi caused an up-regulation of oxidative phosphorylation in the brain (24 genes). There also are strong gene expression differences in brain oxidative phosphorylation associated with behavioral maturation, but the dominant trend is for down-regulation of these genes (Alaux *et al.,* 2009b; Ament *et al.,* 2008). This is puzzling given that *vg* RNAi causes precocious foraging (Nelson *et al.,* 2007); however, JH analog treatment also caused up-regulation of oxidative phosphorylation (Ament *et al.,* 2008). The similar transcriptional effects of JH and Vg highlight the close relationship between these two physiological factors and suggest that additional factors influence whole brain energy metabolism during behavioral maturation.

Vg influences many of the same genes that show changes in brain expression during behavioral maturation, but the direction of these changes were not always the same. This means that Vg influences some gene sets in the brain in a manner consistent with behavioral maturation, and others that are not. Previous analyses have shown that some genes that are differentially expressed in the brain as a function of behavioral maturation are more related to chronological age while others are more related to behavioral state (Whitfield *et al.,* 2006). The gene list for *vg*

RNAi was significantly correlated with age- and behavior-related gene expression changes, showing that Vg shows strong similarities to age- and behavior-related differences, independently, but does not show a strong similarity with behavioral maturation.

Our results also show that the relationship between *vg* RNAi and maturation-related gene lists does not fully reflect the nutritional component of behavioral maturation. Brain gene expression changes due to diet manipulations were not significantly correlated with the *vg* RNAi gene list or the gene list for behavioral maturation. These results are surprising given that Vg is modulated by diet changes (Bitondi & Simoes 1996) and because Vg and diet are good predictors of the transcriptional response associated with behavioral maturation in abdominal tissue (Ament *et al.,* 2011). However, Ament *et al.,* (2011) also showed that the *vg* RNAi transcriptional response is not correlated with diet induced changes in abdominal tissue, suggesting Vg regulates behavioral maturation through gene expression changes that are largely diet independent. Our results similarly suggest *vg* RNAi causes brain gene expression changes that are separate from diet-induced changes, as *vg* RNAi is strongly associated with the age and behavior transcriptional responses but diet is not.

Despite the tight co-regulatory relationship between Vg and JH, the transcriptomic relationship between *vg* RNAi and behavioral maturation apparent from this study does not completely capture the molecular connection between JH and behavioral maturation. The gene list for JHA-treated bees was tightly correlated with *vg* RNAi, age- and behavior-related gene expression changes, as well as behavioral maturation. These results show JH elicits gene expression changes that are associated with behavioral maturation on a larger scale than Vg-related expression

changes – a finding that may reflect a greater proximity of JH to maturation-related transcriptional changes in the brain. The JHA gene list, like the *vg* RNAi gene list, was not correlated with diet changes; thus, JH may also influence behavioral maturation via diet-independent mechanisms. Taken together, these results show that the tight regulatory relationship between JH and Vg is present at the transcriptomic level and that both of these physiological factors act through common pathways to influence brain gene expression.

Recent studies have further explored the relationships between Vg, JH signaling and behavioral maturation. Wang *et al.,* (2012b) found that double RNAi knockdown of *vg* and *usp,* a transcription factor involved in JH signaling, elicited forager-like behavioral and physiological changes, but no changes were elicited by *usp* RNAi alone. Ament *et al.,* (2012b) showed that *usp* knockdown causes nurse-like behavioral and transcriptional changes in abdominal tissue. These two results may reflect regulatory epistatic effects between Vg and USP, such that Vg may influence the *usp* transcriptional cascade to produce different behavioral phenotypes. In our results, *usp* was not differentially expressed in the brain due to *vg* RNAi; however, we did find *fruitless,* a behavior-related transcription factor (reviewed in Robinson *et al.,* 2008; Chandrasekaran *et al.,* 2011) and a direct target of *usp* (Ament *et al.,* 2012b), to be down-regulated due to *vg* knockdown. Down-regulation of *fruitless* is consistent with the expression pattern seen in foragers and JHA-treated bees in abdominal tissue (Ament *et al.,* 2012b). Although the effects of Vg on JH and *usp* signaling should be tested directly, our results suggest that Vg does influence the expression of direct targets of *usp* and it is possible that these changes are mediated by other changes in JH signaling, including changes in the putative binding partner of JH, *methoprene-tolerant* (Charles *et al.,* 2011).

The gene list for *vg* RNAi was most correlated with age-related gene expression changes. This is consistent with Vg's role as an antioxidant and its positive effect on bee longevity (Seehuus *et al.,* 2006). It also is consistent with our finding that the nuclear histone deacetylase, *sirt6,* was among the top predictors of *vg* depletion. *sirt6* is known to regulate lifespan in mice (Kanfi *et al.,* 2012) and recently has been shown to affect the insulin signaling in mice and worms (Sundaresan *et al.,* 2012; Chiang *et al.,* 2012). It is tempting to speculate that there is a mechanistic connection between Vg*, sirt6*, and insulin signaling within the context of honey bee longevity. Insulin signaling has been associated with bee longevity (Corona *et al.,* 2007) and this connection could be manifested in our results through gene expression changes of the *insulin receptor precursor*.

Behavioral maturation in honey bees is a complex process regulated by environmental and physiological factors. It is now evident that many of these factors elicit behavioral changes through changes in gene expression, in both the brain (Whitfield *et al.,* 2006) and peripheral tissues (Ament *et al.,* 2011; Wang *et al.,* 2012a). Our results show this to be the case for vitellogenin and begin to identify the candidate pathways by which this peripheral storage protein exerts its effects. Further work on these mechanisms should help to understand how vitellogenin evolved the novel function of playing a role in the regulation of worker honey bee behavioral maturation.

EXPERIMENTAL PROCEDURES

Bees

The honey bee samples used for this study correspond to the same individuals used in Ament *et al.,* 2011 for fat body transcriptomic analyses. Transcriptomic results from these samples have additionally been used in meta-analyses in Ament *et al.,* 2012a and Ament *et al.,* 2012b but have never before been analyzed in detail. For these samples, colonies were maintained with standard beekeeping practices at the University of Illinois Beekeeping Facility in Urbana IL. To control for genetic variability, bees were obtained from colonies in which each queen was instrumentally inseminated with semen from a single (different and unrelated) male (drone). The experiment was replicated using three genetically distinct colonies. One day-old adult worker bees were obtained from each colony by placing honeycomb frames of emerging brood in a 34˚C incubator. Bees that emerged within a 24-h period were collected and used promptly in the experiments.

Vitellogenin RNA interference

Knockdown of *vitellogenin* (*vg*) (GB13999) was performed by Ament *et al.,* (2011). Double-stranded RNA (dsRNA) probes for *vg* knockdown were generated following Amdam *et al.,*(2006). dsRNA was diluted to a concentration of 10 ng/µl in a buffered saline solution. Each bee was then injected intra-abdominally with 1 µl *vg* dsRNA solution using a microinjection system. Control bees received a 1µl injection of buffered saline solution and untreated control bees received no injection. Bees were paint-marked (Testor's paint, Rockford, IL) on the thorax

according to treatment and placed into Plexiglas cages (10x10x7cm). Each cage contained an equal number of bees from each treatment group (RNAi-injected, saline-injected and untreated bees) and was supplied *ad libitum* with a pollen/honey mixture (45% pollen, 45% honey, 10% water) and a 50% sucrose solution (w/v). Consumption and mortality were monitored daily to ensure there were no measurable differences between cage replicates. Cages were then kept in a 34˚C incubator for a total of 4 days, after which bees were flash-frozen in liquid nitrogen and stored at -80˚C. *vg* knockdown was validated by quantitative real-time PCR. *vg* expression in abdominal tissue was reduced by 56-80 % when compared to saline injected bees, and by 65-84% relative to untreated controls. Honey bee workers have a negligible amount of *vg* mRNA in the head (Corona *et al.,* 2007); therefore, the knockdown effect was not measured in this body region. Samples with the lowest *vg* expression were chosen for the *vg* RNAi treatment group.

RNA preparation and Microarray Hybridization

Bee brains were dissected on dry ice, from partially lyophilized heads, as described in Grozinger *et al.,*(2003). Total RNA was isolated from brain samples using RNeasy Mini Kits (Qiagen, Valencia, CA). The microarray platform was characterized previously in Alaux *et al.,*(2009a). Briefly, the microarray consists of 13,440 distinct oligonucleotide probes based primarily on the honey bee genome sequence (Honey Bee Genome Sequencing Consortium, 2006). Prior to hybridization, samples were amplified using MessageAmp II aRNA Amplification kits (Ambion, Austin, TX). Each amplification reaction was started with 500 ng total RNA and the *in vitro* transcription time for each sample was 5 h. Amplified RNA (2 µg) was then labeled with a Cy3 or a Cy5 dye using the ULS aRNA labeling system (Kreatech Diagnostics, Amsterdam, The

Netherlands). A total of 60 pmol Cy3 and Cy5 labeled material were hybridized onto each microarray. Hybridization was carried out overnight using a 42˚C water bath.

We hybridized a total of 30 samples; these included samples from all three treatment groups, from two honey bee colonies. Samples were hybridized following a loop design similar to that described in Ament *et al.,* 2011. Microarray slides were scanned with an Axon 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA) and analyzed with GenePix Pro 6.0 software (Molecular Devices).

Microarray Data Analysis

To normalize expression intensities, a Lowess transformation was performed with Beehive (http://stagbeetle.animal.uiuc.edu/Beehive). A linear mixed-effects model was used to analyze the log2-transformed fluorescent intensities for each gene. The model accounted for the effects of dye, treatment, individual bee, colony, and microarray. The effects were evaluated with an *F*-test statistic and the *P* values were adjusted for multiple testing using a false discovery rate (FDR) correction. Genes highly expressed in the hypopharyngeal glands, a likely source of contamination in brain samples, were excluded from the gene list (Whitfield *et al.,* 2003).

Functional Enrichment Analyses

Functional assessment of the microarray results was performed with Gene Ontology (GO) enrichment analyses, using *Drosophila melanogaster* orthologs to honey bee genes. Enrichment

of GO terms was assessed using the DAVID Bioinformatic Resources 6.7 functional annotation tool (Dennis *et al.,* 2003). Significant enrichment of GO terms was determined using a hypergeometric test with an FDR correction. The reference gene list consisted of the number of honey bee genes with annotated *Drosophila* orthologs.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was performed to validate some of the microarray results. For each sample, cDNA was synthesized from 200 ng total RNA using the reverse transcriptase enzyme, Arrayscript (Ambion, Austin TX). QPCR was performed using the ABI Prism HT7900 sequence detection system, with SYBR Green detection (Applied Biosystems, Foster City, CA). For technical replication, each sample was run in triplicate on a 384-well plate (Phenix Research Products, Hayward, CA). Relative quantification of the qPCR results was performed using a genomic standard curve. Expression levels for each gene were normalized to a constitutively expressed gene, *rp49* (GB10903)(Grozinger *et al.,* 2003; Ament *et al.,* 2008).

Validation of the microarray results was carried out with samples from both colonies included in the microarray study and from an additional colony. The selection criteria for validation consisted of fold-change differences between the *vg* RNAi and saline-injected treatment groups, and a leave-one-out cross-validation scheme (CMA, R package), which also contrasted these two groups. In total, we attempted to validate the brain microarray results for 6 genes. cDNA synthesis was carried out as outlined above. The gene names and primer sequences used for qPCR analyses are given in Table 2.S1. Statistical analyses for qPCR data were performed using

JMP 7 software (SAS Institute, Cary, NC). A one-way analysis of variance on raw or log-transformed data was used to assess overall significance of the qPCR results. Means separation for each data set was performed with Tukey's HSD mean separation test.

Statistical Analyses to Determine Overlap and Concordance between Gene Lists

To determine whether the overlap between two gene lists ("gene lists" = those genes showing significant differences in expression in each experiment) was significant, we performed Representation Factor analysis (Alaux *et al.,* 2009b). This factor was calculated by dividing the number of observed overlapping genes by the number of estimated overlapping genes that would be expected by chance. The estimated overlap was calculated by multiplying the total number of significant microarray probes in each gene list, divided by the total number of probes analyzed. Statistical significance was assessed using a hypergeometric test (one-tailed). To determine whether the gene expression differences between two gene lists were correlated, we compared the log2 fold change estimates between the two lists and calculated significance using a Pearson's correlation.

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TABLES FOR CHAPTER 2:

Table 2.1. Overlap of differentially expressed genes (FDR <0.05) between *vg* RNAi, JHA, diet and maturation-related experiments. The number of overlapping genes between pairwise comparisons; the number of differentially expressed genes in each experiment is in parentheses; the Representation Factor (RF) is the fold enrichment relative to random; statistical significance (*P*) was calculated using the hypergeometric distribution (one-tailed); (*) denotes RF and associated *P* value was adjusted to compare across different microarray platforms; adjustments were made by using the overlap between differentially expressed oligos in *vg* RNAi, diet and behavioral maturation experiments with the total number of oligos expressed in the JHA experiment.

Experiment	*vg* RNAi – saline (3,138)			Behavioral maturation (1,417)			JHA – control (659)			Age (Old – Young) (875)			Behavior (Forager–Nurse) (1,037)		
	Overlap	RF	*P* value	Overlap	RF	*P* value	Overlap	RF	*P* value	Overlap	RF	*P* value	Overlap	RF	*P* value
***vg* RNAi – saline (3,138)**	-	-	-	526	1.484	<0.0001	273	1.180*	0.0001	297	1.357	<0.0001	337	1.299	<0.0001
Behavioral maturation (1,417)	526	1.484	<0.0001	-	-	-	139	1.560*	<0.0001	502	5.384	<0.0001	561	5.076	<0.0001
Protein poor- rich (372)	156	1.618	<0.0001	91	2.091	<0.0001	55	2.218*	<0.0001	54	2.01	<0.0001	70	2.198	<0.0001
JHA – control (659)	273	1.180*	0.0001	139	1.560*	<0.0001	-	-	-	86	1.782*	<0.0001	93	1.621*	<0.0001

Table 2.2. Genes showing concordant gene expression changes between pairwise comparisons. Number and percentage of genes showing directionally concordant transcriptional responses based on the predicted effects of each treatment on behavioral maturation. Percentages are based on the number of overlapping genes in each pairwise comparison (see Table 2.1).

Experiment	*vg* RNAi – saline		Behavioral maturation		JHA – control		Age (Old – Young)		Behavior (Forager–Nurse)	
	# genes	%	# genes	%	# genes	%	# genes	%	# genes	%
***vg* RNAi – saline**	-	-	229	43.536	149	54. 579	189	63.636	192	56.973
Behavioral maturation	229	43.536	-	-	98	70.503	502	100.00	561	100.00
JHA - control	149	54. 579	98	70.503	-	-	55	63.953	71	76.344
Protein poor- rich	77	49.359	54	59.341	21	38.181	41	75.926	34	48.571

Table 2.3. Pairwise correlations for brain transcriptional profiles associated with *vg* RNAi, JH, diet and behavioral maturation-related experiments. Pearson's correlations and *P* values are shown for genes overlapping across pairwise comparisons. Gene lists for each experiment are comprised of genes significantly different using a FDR <0.05.

Experiment	*vg* RNAi – saline		Behavioral maturation		JHA – control		Age (Old – Young)		Behavior (Forager–Nurse)	
	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*
***vg* RNAi – saline**	-	-	-0.084	0.053	0.135	0.026	0.209	0.0003	0.146	0.007
Behavioral maturation	-0.084	0.053	-	-	0.283	0.0007	0.964	<0.0001	0.954	<0.0001
Protein poor- rich	0.0466	0.564	0.081	0.445	-0.259	0.056	0.114	0.412	0.037	0.759
JHA – control	0.135	0.026	0.283	0.0007	-	-	0.300	0.005	0.384	0.0001

FIGURES FOR CHAPTER 2:

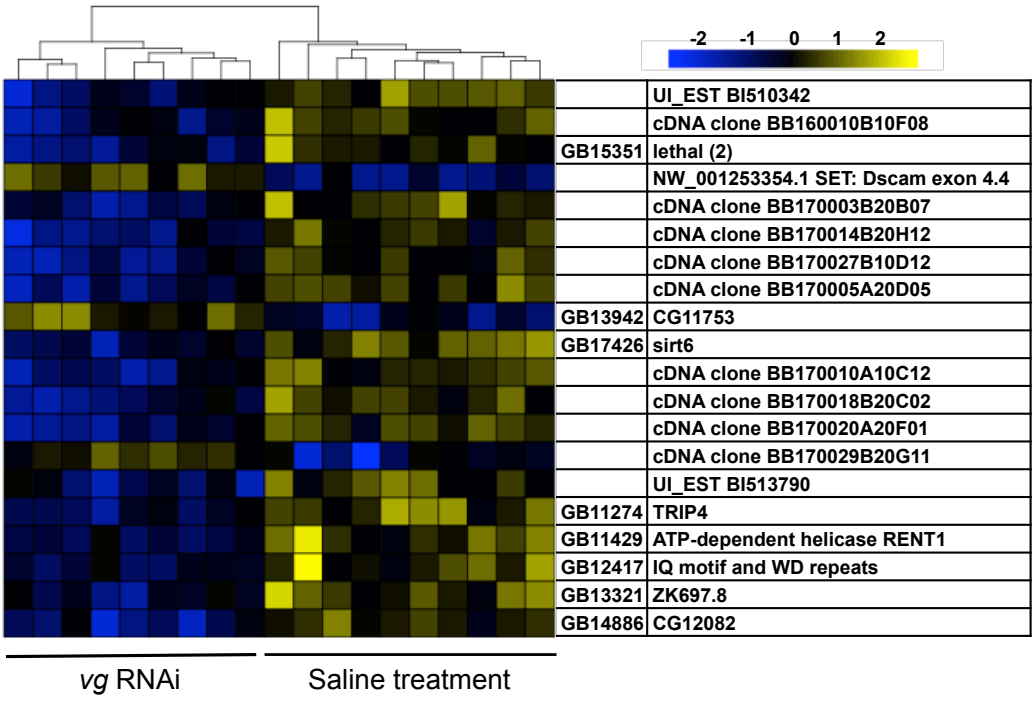

Figure 2.1: Heat map of expression differences for genes predictive of *vg* RNAi treatment. Top predictor genes were identified using class prediction analyses and are ordered based on their ranking as a classifier. Gene names shown are based on the *Apis mellifera* Official gene set 2 (Honey bee Genome Sequencing Consortium, 2006) and their orthology to *Drosophila melanogaster*. The median-centered log2 expression estimates are shown for *vg* RNAi and saline injected bees. Yellow represents up-regulation and blue represents down-regulation.

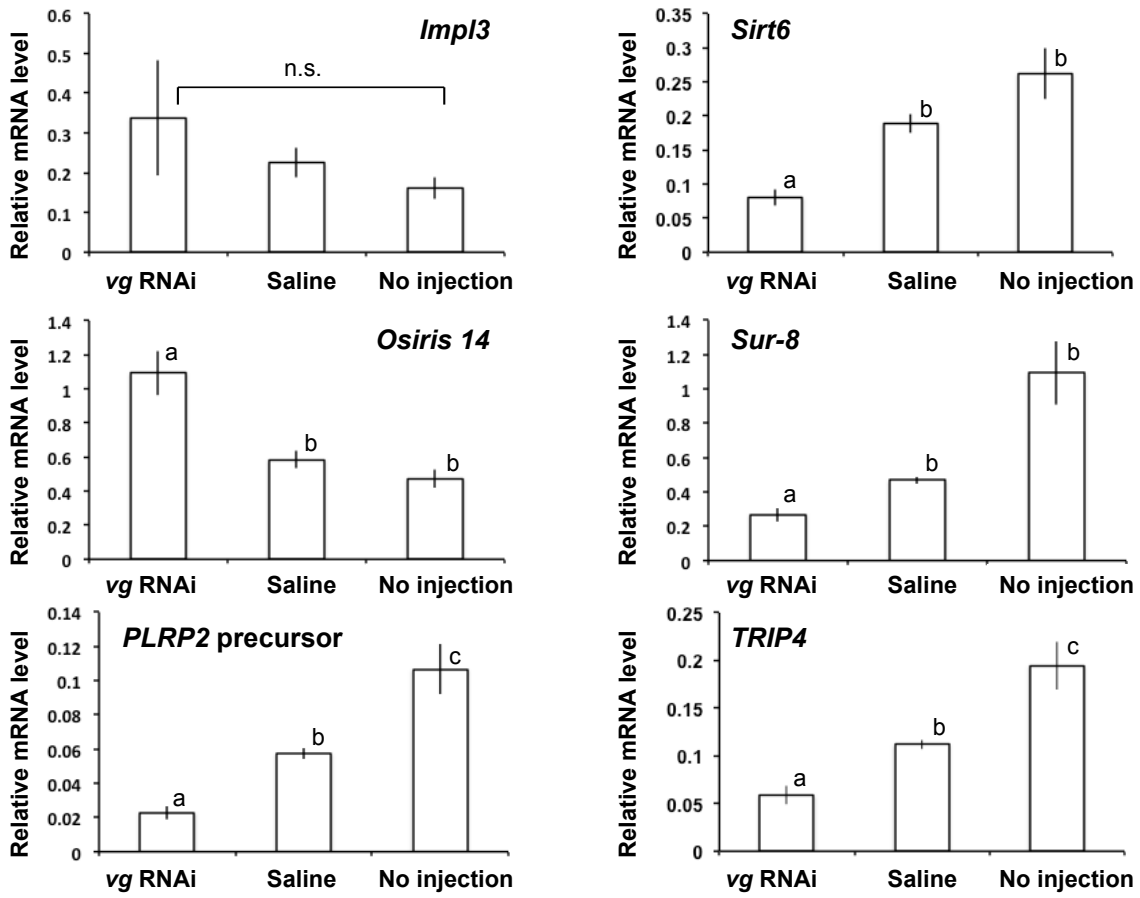

Figure 2.2: Validation of the microarray results using qPCR. qPCR results for *vg* RNAi, saline and no injection controls are shown for 6 genes. The mRNA levels shown are relative to the control gene, *rp49*. Values for each group are means ± S.E.M. for 3 colony replicates (n=24); significant differences between treatment groups are denoted by letters (a, b, c).

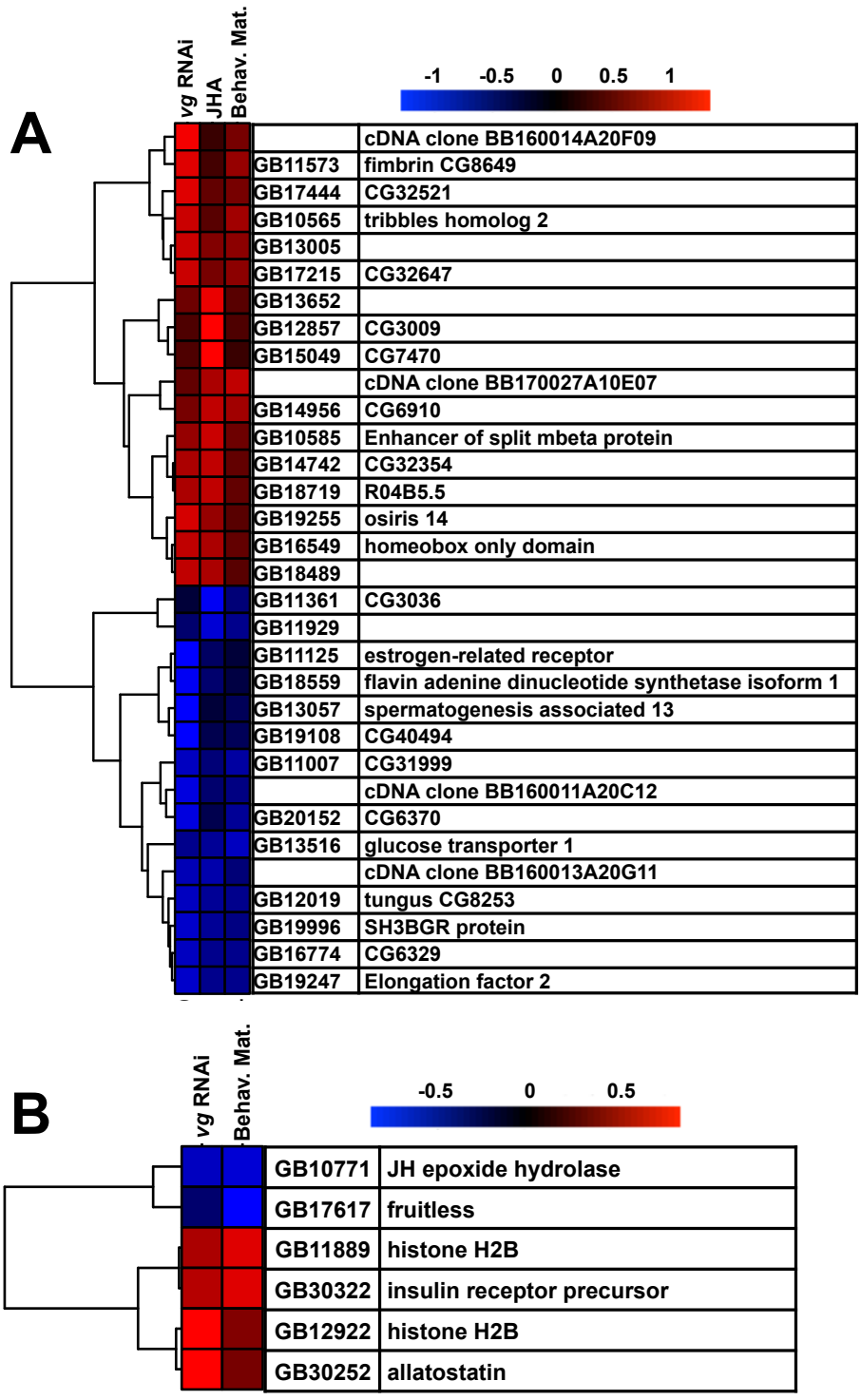

Figure 2.3: Genes showing concordant responses across gene lists associated with *vg* RNAi, JHA treatment and behavioral maturation. (A) log2 fold changes for differentially expressed

genes (FDR<0.05) that show concordant responses relative to the predicted effects of each factor on behavioral maturation. Experimental contrasts in each experiment are: *vg* RNAi – saline, JHA – control, and forager – nurse for behavioral maturation. (B) log2 fold changes for selected genes showing a concordant response between *vg* RNAi and behavioral maturation. In both A and B, gene names are shown based on the *Apis mellifera* Official gene set 2 (Honey bee Genome Sequencing Consortium, 2006) and their respective *Drosophila melanogaster* ortholog. Red depicts up-regulation and blue represents down-regulation.

SUPPLEMENTARY MATERIAL FOR CHAPTER 2:

Table 2.S1. Primer sequences used for qPCR validation

Gene Name (abbreviation)	Accession No.	Forward Primer	Reverse Primer
Osiris 14	GB19255	TCTCCGTCAAGACTGTCAGCA	GATGTCAATGTCGCTGGACCT
Ecdysone-inducible gene L3 (Impl3)	GB13882	CTGTCTTTGCCCTGCTCATTG	GTGCGTCTCCTCCTCCGTTA
Leucine-rich repeat protein soc-2 homolog (Sur-8)	GB16542	TCTCAATTACCAGATGGACTACTTGC	AAGCATTCCGAGATAATGTGATTGT
Ribosomal protein 49 (Rp49)	GB10903	GGGACAATATTTGATGCCCAAT	CTTGACATTATGTACCAAAACTTTTCT
Pancreatic lipase-related protein 2 precursor (PLRP2)	GB30584	ATTTGTGGTTTTGCTGCGAAA	GGTTGTGCAGGATCTAGCCCT
Sirt6 histone deacetylase (Sirt6)	GB17426	TCGCGACTAAGAGCGTAGGAA	TCCACGGCATGGACGTC
Thyroid hormone receptor interactor 4 (TRIP4)	GB11274	TCATGGTATTCATCACATAGAGGAAGA	TTGGTGTTTTAGATGTTGCAGCA

Table 2.S2. Pairwise correlations between genes differentially expressed (FDR<0.01) in *vg* RNAi, JH, diet and behavioral maturation-related experiments. Pearson's correlations and *P* values are shown for genes that overlap across pairwise comparisons.

Experiment	*vg* RNAi – saline		Behavioral maturation		JHA – control		Age (Old – Young)		Behavior (Forager–Nurse)	
	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*
***vg* RNAi – saline**	-	-	-0.042	0.550	0.197	0.034	0.247	0.022	0.199	0.017
Behavioral maturation	-0.042	0.550	-	-	0.322	0.007	0.973	<0.0001	0.973	<0.0001
Protein poor- rich	0.293	0.155	-0.170	0.427	-0.712	0.009	-0.189	0.517	0.042	0.861
JHA – control	0.197	0.034	0.322	0.007	-	-	0.376	0.044	0.452	0.002

Table 2.S3. Pairwise correlations between all genes expressed in *vg* RNAi, JH, diet and behavioral maturation-related experiments. Pearson's correlations (r) and related *P* values are shown for genes overlapping across pairwise comparisons.

Experiment	*vg* RNAi – saline		Behavioral maturation		JHA – control		Age (Old – Young)		Behavior (Forager–Nurse)	
	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*	Correlation (r)	*P*
***vg* RNAi – saline**	-	-	-0.021	0.021	0.051	0.002	0.089	<0.0001	0.052	<0.0001
Behavioral maturation	-0.021	0.021	-	-	0.099	<0.0001	0.708	<0.0001	0.664	<0.0001
Protein poor- rich	-0.027	0.004	-0.004	0.689	-0.075	<0.0001	0.074	<0.0001	-0.051	<0.0001
JHA – control	0.051	0.002	0.099	<0.0001	-	-	0.049	0.002	0.161	<0.0001

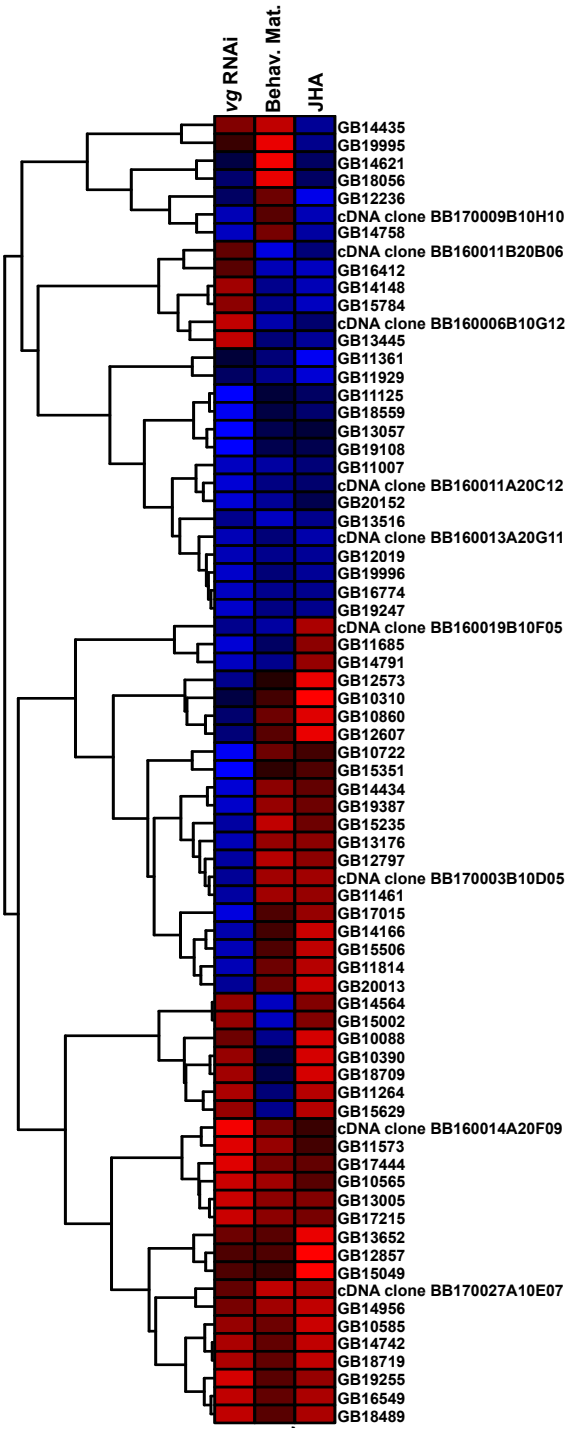

Figure 2.S1: Genes overlapping between gene lists associated with *vg* RNAi, JHA treatment and behavioral maturation. log2 fold changes for differentially expressed genes (FDR<0.05)

that overlap across *vg* RNAi, JHA treatment and behavioral maturation microarray experiments. Experimental contrasts in each experiment are: *vg* RNAi – saline, JHA – control, and forager-nurse for behavioral maturation. Gene names are shown based on the *Apis mellifera* Official gene set 2 (Honey bee Genome Sequencing Consortium, 2006). Red depicts up-regulation and blue represents down-regulation.

CHAPTER 3:
EFFECTS OF DIET AND JUVENILE HORMONE ON MATURATION-RELATED GENE EXPRESSION CHANGES IN THE *PARS INTERCEREBRALIS* OF THE HONEY BEE BRAIN[2]

ABSTRACT

Insect endocrine systems are composed of brain neurosecretory cells that innervate endocrine glands posterior to the brain. These nervous and neuroendocrine systems mediate environmental conditions to control a variety of life history traits. In this study, my goal was to provide mechanistic insights as to how neurosecretory signals mediate division of labor among workers in honey bees (*Apis mellifera*). Worker division of labor is a maturational process in which individuals transition from performing in-hive tasks to foraging for food outside the hive. Social and nutritional cues converge on endocrine factors to regulate worker maturation but whether neurosecretory systems are central to this process is not known. To explore this, I performed targeted transcriptomic profiling of a neurosecretory region of the brain known as the *pars intercerebralis* (*PI*). I first compared *PI* transcriptional profiles for bees performing in-hive tasks and bees engaged in foraging. Using these results as a baseline, I then performed manipulative experiments to test whether the *PI* is responsive to dietary changes and/or changes in juvenile hormone (JH) levels that occur prior to foraging onset. Results reveal a robust molecular signature of maturation in the *PI*, and a subset of these changes was consistent with transcriptomic changes elicited by JH analog treatment. This result, together with *cis* motif enrichment analyses, suggests the *PI* may mediate worker division of labor through transcriptomic changes induced by JH. In contrast, dietary changes did not induce gene

[2] This chapter is in preparation for publication. Co-authored with Seth A. Ament, Sandra L. Rodriguez-Zas, Bruce R. Southey and Gene E. Robinson.

expression changes in the *PI* consistent with behavioral maturation or JH treatment. From these results I propose a new verbal model in which division of labor in honey bee workers is regulated primarily through social changes that are independent of diet. I propose that the relationship between diet and nutritional physiology is attenuated in worker bees and that in its place is a novel relationship between social signals and nutritional physiology that is mediated by JH.

INTRODUCTION

A major goal in biology is to understand the mechanisms that underlie complex traits including animal social behavior (Robinson et al., 2008). Towards this end, social insects provide tractable systems in which social behavior can be dissected at molecular and physiological levels. Honey bees (*Apis mellifera*) in particular have become social model organisms for studying how extrinsic and intrinsic factors shape insect societies (Smith et al., 2008; Toth and Robinson, 2007).

Honey bee colonies are extremely complex: in addition to a reproductive division of labor (queens and workers), honey bee colonies exhibit a partitioning of tasks within the worker caste. Adult workers divide labor through a form of behavioral maturation. They perform in-hive tasks such as brood care ("nursing") for the first 2-3 weeks of life, and then switch to foraging tasks outside (Winston, 1987). This behavioral maturation has been used extensively to understand the mechanisms that regulate social insect division of labor.

Research has shown that the typically age-related process of behavioral maturation is not rigid and is regulated by social and nutritional cues. For example, the depletion of a colony's food stores can accelerate maturation and induce early foraging (Schulz et al., 1998), while treatment with queen mandibular pheromone (QMP) — a queen-produced substance known to suppress worker ovary development — delays foraging onset (Pankiw et al., 1998). Notably, behavioral maturation is associated with dietary changes as well as changes in nutritional physiology that occur prior to foraging onset. For example, young hive bees feed on pollen and have high levels of abdominal lipids, while older foraging bees mostly feed on honey and nectar and have low levels of abdominal lipids (Crailsheim et al., 1992; Toth and Robinson, 2005).

These environmental cues regulate foraging onset through changes in brain expression and key physiological factors downstream. Thousands of brain gene expression changes are associated with behavioral maturation (Alaux et al., 2009a; Whitfield et al., 2003) and a subset of these is regulated by social and nutritional cues (Grozinger et al., 2003; M. M. Wheeler et al., 2013). These transcriptomic changes are thought to converge on hormonal signals including the pleiotropic hormone, juvenile hormone (JH).

JH plays a prominent role in worker division of labor. JH levels increase during behavioral maturation (Huang et al., 1991; Huang and Robinson, 1995; Rutz et al., 1976) and several manipulative experiments, including removal of the endocrine glands that produce JH, have demonstrated that high JH titers increases a bee's likelihood to become a forager (Robinson, 1987; 1985; Sullivan et al., 2000). JH is known to have co-regulatory interactions with peripheral nutritional signals related to an individual's lipid stores and is known to elicit brain gene

expression changes that are consistent with behavioral maturation (Corona et al., 2007; Guidugli et al., 2005; Whitfield et al., 2006). Moreover, recent bioinformatic analyses of brain transcriptomic data implicate transcription factors related to JH-signaling as key regulators of behavioral maturation (Ament et al., 2012a). These results indicate that environmental and physiological factors interact with the brain to regulate behavioral maturation. The goal of my study was to develop a more complete understanding of these interactions by studying a brain region known for controlling a broad array of life history traits in other insects and with structural and functional connections to JH.

In insects, hormones such as JH are neurohormones produced in endocrine glands posterior to the brain. These endocrine glands are innervated by projections extending from neurosecretory cells in the brain (Eichmüller et al., 1991; Ludwig et al., 2002). The majority of these neurosecretory cells are found in a brain region known as the *pars intercerebralis* (*PI)*. Together, these neurosecretory and endocrine systems adjust physiological and behavioral responses through the production of neuropeptides and hormones, such as insulin and JH, to control a wide array of processes including longevity, reproduction and diapause (Flatt et al., 2005; Hodkova, 1976; Shiga and Numata, 2000). In this study, I hypothesized that the *PI* also plays a role in worker division of labor by responding to nutritional signals and to JH through transcriptional changes that influence other neural and neuroendocrine systems.

The *PI* is histologically recognized as the dorsal medial domain of the insect brain. It is located anterior to the calyces of the mushroom bodies and dorsal to the central complex (Ludwig et al., 2002; Strausfeld, 1976). I tested whether the *PI* is involved in behavioral maturation by

dissecting this brain region using laser capture microdissection and performing subsequent transcriptomic analyses. I first generated *PI* gene expression profiles for nurse and forager bees. Using these results as a baseline, I then generated gene expression profiles for bees fed either a high protein or a diet with no protein diet and treated with the JH analog (JHA) methoprene or a solvent control. Because these diet and JHA treatments mimic physiological changes that occur naturally during behavioral maturation, I was able to test two predictions based on the hypothesis that the *PI* is involved in regulating behavioral maturation. If this hypothesis is correct, then the *PI* should be transcriptomically responsive to nutritional signals and/or to endocrine signals in a manner consistent with transcriptomic changes that occur during behavioral maturation. The results of this study implicate for the first time the *PI* in the regulation of honey bee behavioral maturation, and provide evidence for changes in *PI* function associated with honey bee social evolution.

MATERIALS AND METHODS

Behavioral collections, JHA treatments and diet manipulations

Honey bees (*Apis mellifera ligustica*) were collected from colonies maintained according to standard beekeeping practices at the University of Illinois Bee Research Facility, Urbana, IL, USA. To partially control for genetic variation, I used colonies derived from queens that were instrumentally inseminated with semen from a single male. I collected nurses and foragers using standard behavioral assays (Ament et al. 2008). Briefly, to collect nurse bees I opened the colony brood chamber and collected bees that inserted their head into honeycomb cells

containing larvae. I collected returning foragers at the colony entrance when visible pollen loads were present on their hind legs. Field collections were performed in the summer of 2010.

JHA and diet manipulation experiments were performed with bees that were 7-9 days old. To collect this focal group, I removed frames of dark-eyed pupae from colonies and placed them in a 34°C incubator with 80% RH. Bees that emerged from these frames were marked with a dot of colored paint (Testor's Paint, Rockford, IL, USA) and returned to their natal colony. I repeated this process for three consecutive days to obtain a large base population (> 1000 bees) within colonies. After 9 days, I collected marked bees from colonies and placed them into Plexiglas cages (25-30 bees per cage) for JHA treatments and diet manipulations.

JHA and diet manipulations were set-up as 2*x*2 experiment. I topically treated individuals with the JHA methoprene (200 µg in 5µl of acetone) or with 5µl of acetone as a control (Robinson, 1987). After treatment, both JHA-treated and control bees were placed into Plexiglas cages supplied with either a high protein diet (45% pollen, 45% honey, 10% water) or a diet with no protein (50% sucrose w/v) *ad libitum* (Ament et al., 2011; 2008). Cages were kept in a 34°C incubator with approximately 30% RH for four days. Consumption and mortality were monitored daily. I performed two independent trials of this experiment with unrelated honey bee colonies. These colonies also were unrelated to colonies used in the nurse-forager experiment described above. The JHA and diet manipulation experiments were performed in the summer of 2011.

Laser Capture Microdissection

To collect cells from the *PI,* I first anesthetized bees using CO_2 and then immobilized them further on wet ice. I then dissected bee brains from each individual using cold bee saline (Bicker, 1996) and embedded each brain into small tissue molds containing O.C.T. medium (Tissue-Tek®). I embedded each brain dorsal side down and froze each embedded brain immediately using dry ice. Samples were placed at -80°C for long-term storage.

I performed laser capture microdissection (LCM) by sectioning frozen brains into 35 µm sections using a cryostat (Leica Biosystems®). A total of 7-10 dorsal sections were collected onto PEN Membrane Glass slides (Applied Biosystems®) and stained using Histogene® LCM Frozen Section Staining Kit (Applied Biosystems®). Cells from the *PI* were captured using the ArturusXT ™ LCM instrument. I captured cells along the midline of the brain from all sections dorsal to the central body (Eichmüller et al., 1991). Cells from these sections were collected onto 2-3 Capsure® HS LCM caps (Applied Biosystems®). Extraction buffer (15µl; PicoPure ™ RNA Isolation Kits (Applied Biosystems®) was applied directly onto each cap and incubated for 30 min at 42°C. After incubation, I eluted each sample using a centrifuge and stored each sample aliquot at -80°C for subsequent RNA isolation.

RNA extraction and Amplification

RNA from each *PI* sample was extracted using PicoPure ™ RNA Isolation Kits (Applied Biosystems®) following the manufacturer's guidelines for LCM tissue. After extraction, RNA

from each sample was amplified using two rounds of linear antisense RNA (aRNA) amplification (Van Gelder et al., 1990). *In vitro* transcription was performed using the MEGAscript® T7 Kit (Invitrogen™) and carried out for 14 h for the first amplification round and 6 h for the second round of amplification. aRNA quantity was assessed on a spectrophotometer and aRNA quality was assessed using an Agilent 2100 Bioanalyzer.

Microarrays

Nurse and forager *PI* samples were hybridized onto microarrays for gene expression profiling. The microarray platform was characterized previously by Alaux et al. (2009a) and consists of 13,440 oligonucleotide probes based primarily on the honey bee genome sequence v 4.0 (Honeybee Genome Sequencing Consortium, 2006). Prior to hybridization, 2 μg of each sample were labeled with a Cy3 or a Cy5 dye using the ULS aRNA labeling system (Kreatech Diagnostics). 60 pmol of each labeled sample were hybridized onto each microarray. Hybridization was carried out overnight using the MAUI® Hybridization System (BioMicro Systems Inc). Samples were hybridized onto each microarray following a loop design (Figure 3.S1). In total, I hybridized 32 samples, 8 per behavioral group per colony. Microarray slides were scanned with an Axon 4000B Microarray Scanner (Molecular Devices) and analyzed with GENEPIX Pro 6.0 software (Molecular Devices).

Microarray data analysis

Expression intensities were normalized with a Lowess transformation with BEEHIVE (http://stagbeetle.animal.uiuc.edu/beehive.html). A linear mixed-effects model that accounted for the effects of dye, treatment, colony and individual bee was used to analyze the log2-transformed fluorescent intensities for each gene. The results were evaluated with an *F*-test statistic and FDR corrected *P*-values. Array probes were updated to the new version of the *Apis mellifera* genome, Assembly 4.5 (Elsik et al., 2014), by mapping array probes using Bowtie 2.

During the course of my analyses, I observed that seven major royal jelly protein genes were differentially expressed in *PI* samples of nurses and foragers, with substantial log-fold change differences. These genes are differentially expressed, and at high levels, in the hypopharyngeal glands (HPG), which surround the brain. To minimize the possibility that my results were affected by small amounts of HPG tissue, I excluded a set of 113 genes expressed at high levels in the HPG (Whitfield et al., 2003). Expression of a few major royal jelly protein genes has been found in the brain (Peixoto et al., 2009) but without further validation I cannot determine if these genes were expressed in the *PI* or the HPG.

cDNA libraries and RNA-sequencing

Gene expression profiles for *PI* samples from JHA and diet-treated bees were generated using RNA-sequencing (RNA-seq). cDNA libraries from each *PI* sample were generated from 400 ng of aRNA with the TruSeq RNA Sample Preparation Kit (Illumina). Library concentrations were quantified using Qubit® fluorometric quantitation and quantitative real-time PCR (qPCR) using Kapa Library Quant kits (KapaBiosystems). Average fragment size and overall quality were

evaluated with the Agilent 2100 Bioanalyzer platform and an Agilent High Sensitivity DNA kit. For sequencing, all libraries were diluted to a 10nM concentration. In total, I sequenced 32 libraries (8 libraries per treatment group, 4 from each colony replicate). Eight libraries were sequenced per lane, with 2 samples from each treatment group per lane. cDNA libraries were sequenced as single-end, 100 nt reads, using the Illumina HiSeq2000.

RNA-seq data analysis

RNA-seq generated an average of 23,833,961 reads per cDNA library. Reads from each library were aligned to the *Apis mellifera* genome, Assembly 4.5 (Elsik et al., 2014) using Tophat (Trapnell et al. 2008). Approximately 80% of reads within each library mapped to the honey bee genome. Aligned read counts for each gene were counted using the program HTSeq v0.5pv2 (http://www-huber.embl.de/users/anders/HTSeq) and the *Apis mellifera* Official Gene Set, v3.2. Reads that did not map uniquely or that mapped to genomic locations outside genes were not included in subsequent analyses for differential expression. Differential expression was assessed using a negative binomial regression and generalized linear model with count data normalized by library size and library composition. Dispersion estimates for each individual gene were used to calculate differential expression. The model tested for the main effect of JHA treatment, the main effect of diet and an interaction between these two factors. Significance for these main effects was assessed using FDR corrected *P*-values (FDR<0.1). Significance for pairwise contrasts was assessed using raw *P*-values ($P<0.001$) and assessing whether genes were significant due to JHA treatment, diet or the interaction between these to factors.

Bioinformatic analysis of transcription factors and enrichment of cis *regulatory motifs*

To identify all the transcription factors that were differentially expressed in my experiments, I drew upon a comprehensive list of transcription factors that was compiled previously (Chandrasekaran et al., 2011). I updated this list with information from the latest official honey bee gene set (v3.2)(Elsik et al., 2014).

To determine whether my lists of differentially expressed genes suggest the involvement of specific transcription factors as regulators, I determined whether my lists were enriched for particular *cis* regulatory motifs, using the Motif Enrichment Tool (http://veda.cs.uiuc.edu/regSetFinder/interface_help.html). This bioinformatic tool predicts transcription factor regulators by finding significantly associated sets of genes that share a transcription factor motif in the their promoter regions. Genes thus targeted by a transcription factor motif were determined for the *Apis mellifera* genome (v4.5) and the official gene set (v3.2) following methods similar to Alaux et al. (2009b). I analyzed 5 Kbp of sequence upstream of each gene's transcriptional start site. I tested for significant associations between sets of upregulated genes and specific *cis* regulatory motifs with hypergeometric tests with an FDR correction. To test whether *cis* motifs were significantly associated across multiple gene lists, I followed the methods in Alaux et al. (2009b). I looked for significant *cis* motifs (raw $P<0.05$) that occurred on different gene lists and calculated the combined *P*-value for each motif.

RESULTS

Gene expression differences associated with behavioral maturation in PI

Microarray profiling of the *PI* revealed extensive differences in gene expression between nurses and foragers (2,663 genes, ANOVA, FDR<0.1). The magnitude of gene expression differences in the *PI* is in the same range as previously reported for whole brain microarray comparisons of nurses and foragers (Alaux et al., 2009a). Moreover, 22% (589 genes) of the genes differentially expressed between nurses and foragers in the *PI* were also differentially expressed in the whole brain (Figure 3.1A). Correlation analysis of the log-fold changes of genes overlapping between Alaux et al. (2009a) and the present study revealed that the majority of genes were concordant in their direction of change (Figure 3.1B).

Gene Ontology enrichment analyses showed transcripts differentially expressed in the *PI* of nurses and foragers were enriched for terms related to energy metabolism and protein turnover (Table 3.S1); genes upregulated in foragers were specifically enriched for terms such as Detection of Light Stimulus and the Oxidative Phosphorylation Pathway, while genes upregulated in nurses were enriched for the terms Ribosome and Translation. Genes differentially expressed between nurses and foragers in both the *PI* and the whole brain were related to Translation, Mitochondrial Processes and Branched Amino Acid Degradation (Figure 3.1A, Table 3.S2). A finer-grained analysis, using only the genes differentially expressed in both the *PI* and the whole brain and with directionally concordant log-fold changes (323 genes), showed these genes were enriched for protein turnover (Ribosome, 28 genes, Benjamini = 2.39E-19). Changes in energy metabolism were not enriched in the concordant gene set, indicating they were in the opposite direction in whole brain (downregulated in foragers)

compared to the *PI* (upregulated in foragers). Genes only differentially expressed in the *PI* were related to Response to Radiation, Translation Factor Activity, Mitochondrial Envelope and Amine Transport, while genes only differentially expressed in whole brain were related to Glycoprotein Biosynthesis (Figure 3.1A, Table 3.S2).

As the *PI* contains neurosecretory cells, I expected to detect differentially expressed neuropeptide genes that had been undetected in prior whole brain analyses. I found a total of 10 differentially expressed neuropeptide genes in the *PI* of nurse and forager bees (Figure 3.1C); six genes had been detected in whole brain and four had not. Neuropeptide genes that were only differentially expressed in the *PI* encode *neuropeptide F*, *orcokinin, sulfakinin* and *tachykinin.* The neuropeptide genes that overlapped between both datasets showed similar upregulation in foragers with the exception of *adipokinetic hormone,* which was downregulated in forager whole brain and upregulated in forager *PI*.

Effect of diet and JHA treatment on gene expression in the PI

Analyses of RNA sequencing results revealed that the overall effect of diet manipulations (without controlling for JHA treatment) did not significantly influence gene expression in the *PI* (1 differentially expressed gene, FDR<0.1, Table 3.1). By contrast, the overall effect of JHA treatment (without controlling for diet manipulations) caused changes in hundreds of genes (365 differentially expressed genes, FDR<0.1). A total of 17 genes (FDR<0.1) showed a significant interaction between JHA and diet (Table 3.1). Diet *x* JHA pairwise comparisons show a total of 62 and 73 genes to be differentially expressed in diet-based comparisons and between 110 to 359

genes to be differentially expressed in JHA-based contrasts (Table 3.1). For diet-based comparisons, approximately 28 and 40% of differentially expressed genes were differentially expressed as a main effect of JHA treatment or due to the interaction between diet and JHA treatment (Table 3.1). For JHA-based contrasts approximately 55-70% of differentially expressed genes in each list were differentially expressed due to the main effect of JHA treatment and less than 5% could be explained by an interaction between diet and JHA treatment. These results all emphasize that the effect of JHA treatment on *PI* gene expression is much more significant than the effect of diet manipulations.

Functional GO analyses performed for the gene lists generated by the pairwise comparisons yielded significant enrichment for diet-treated bees (Ctrl P^- vs. Ctrl P^+) and the JHA^+P^- vs. Ctrl P^- comparisons; both of these contrasts were enriched for differences in cytochrome P450 expression. The JHA^+P^- vs. Ctrl P^- comparison also was associated with Protein Folding (heat shock proteins), translation (Ribosome) and Epidermal Growth Factor domain (Table 3.S3).

Diet and JHA manipulations influenced the expression of 3 neuropeptide genes, including *corazonin* (GB53951), *LRNQLDIGDLQ-containing* (GB43119) and *NVPIYQEPRF-containing* (GB44988). *Corazonin* was upregulated in pairwise contrasts that included JHA^+P^+, while *LRNQLDIGDLQ-containing* was upregulated in pairwise contrasts that included JHA^+P. *NVPIYQEPRF-containing* was upregulated in bees that received a high-protein diet. Interestingly, the *NVPIYQEPRF-containing* gene was also differentially expressed in the *PI* of nurse and foragers but was upregulated in foragers relative to nurses (even though nurses have a diet richer in protein than foragers).

Regulatory changes associated with behavioral maturation and the PI

To understand whether diet and JHA treatment manipulations induced gene expression changes that were overall consistent with behavioral maturation, I performed gene-wise comparisons between the *PI* nurse vs. forager dataset and each diet *x* treatment combination. Between 27 to 49% of the genes differentially expressed in pairwise comparisons overlapped with the nurse-forager results (Figure 3.2A). Correlations for log-fold changes of these overlapping genes show no significant correlation between behavioral maturation and diet manipulations (Figure 3.2B). In contrast, there were significant correlations between behavioral maturation and JHA treatments, in the expected direction. For example, the ortholog of the cytochrome P450 gene, *Cyp4g1*, was upregulated in foragers compared to nurses and upregulated by JHA treatment in the $JHA^{+}P^{-}$ vs. Ctrl P^{-} contrast.

The results of these comparisons suggested that differences in endogenous JH levels could be driving the nurse-forager differences I detected in the *PI*; nurses have low blood titers and foragers high titers (Huang et al., 1991). I therefore explored to what extent these shared gene expression changes were elicited by similar changes at the regulatory level. First, I looked for transcription factors that were differentially expressed in nurses vs. foragers and as a function of JHA treatment. I found a total of 82 differentially expressed transcription factors in the *PI* of nurses and foragers (Table 3.S4). These included *ftz-f1*, *fruitless*, *Xpb1, Creb*, *Deaf1,* and NFkβ*;* all of which have been proposed as major regulators of honey bee behavioral maturation (Chandrasekaran et al., 2011). Only 7 transcription factors were differentially expressed in JHA

pairwise contrasts, and of these 2 overlapped with the *PI* nurse vs. forager data set (GB50553, GB55635, Table 3.S5). Notably the JH-related transcription factor, *kr-h1* was differentially expressed in two of the pairwise contrasts but was not differentially expressed in the *PI* nurse vs. forager data set.

Next, I performed *cis* regulatory motif analyses to indirectly identify transcription factors contributing to differential expression in my experiments. Genes upregulated in the *PI* of nurse bees were significantly enriched (FDR<0.1) for 9 *cis* motifs (Table 3.S6) including the *cis* motif for the JH-related transcription factor, *usp*. No *cis* motifs were enriched for genes upregulated in the *PI* of forager bees. In diet *x* treatment comparisons, I found significant *cis* motif enrichment for one gene list: genes upregulated by JHA treatment in the JHA+P- vs. Ctrl P- comparison were enriched for 4 *cis* motifs, including the motif for NFkβ and the NFkβ-related heterodimers, DIF and relish (Table 3.S7).

To compare across nurse vs. forager and diet *x* JHA experiments, I identified *cis* motifs ($P<0.05$) that overlapped between experiments. I found 5 *cis* motifs associated with genes upregulated in nurse bees and genes upregulated in acetone-treated bees (Ctrl bees) (Table 3.2). These were indicative of changes in endocrine signaling and included the heterodimer, NR1H2-RXRA, which contains an isoform of the vertebrate homolog of *usp*, RXR (Oro et al., 1990). Overlapping *cis* motifs for genes upregulated in forager bees and JHA treated bees indicate both of these gene lists were associated with the NFkβ and the heat shock factor, HSF.

I also looked for an overlap between transcription factors that were differentially expressed between nurses and foragers and transcription factors derived from my *cis* motif analyses. I found several transcription factors that met these criteria, including *fruitless, Deaf1, HLH54F, ATF-2* and NFkβ, suggesting these are major regulators of changes in the *PI* related to behavioral maturation (Table 3.S8). I then examined the extent to which JHA-treatment could explain the regulatory changes associated with behavioral maturation. I used the transcription factor list from the nurse vs. forager experiment and looked for overlap with the JHA-based *cis* motif analyses. I found 3 transcription factors that met these criteria; these were *Tkr*, *HLH54F* and *crc*. All three were associated with genes upregulated by JHA treatment.

DISCUSSION

In this study, I performed gene expression profiling of the *PI* to explore whether this neurosecretory brain region might play a key role in honey bee behavioral maturation. My results reveal a robust molecular signature of behavioral maturation in the *PI*. Moreover, a subset of maturation-related differences in the *PI* was shared with transcriptomic changes associated with JH, a known regulator of honey bee behavioral maturation. These results support my hypothesis that the *PI* interacts with JH signaling related to behavioral maturation. A surprising aspect of my results is that there is limited evidence to support my other hypothesis, that the *PI* is responsive to dietary changes related to behavioral maturation.

The molecular signature of behavioral maturation in the *PI* was related to changes in protein turnover, heat shock protein genes (Response to Radiation) and energy metabolism. In particular,

I found that protein translation was upregulated in the *PI* of nurses compared to foragers and that this upregulation was consistent with previous findings from an analogous whole brain study (Alaux et al., 2009a). These results may reflect changes in neural plasticity that occur during behavioral maturation, as this process occurs in several regions throughout brain (Withers et al., 1993). However, many of the nurse-forager expression differences in the *PI* were not detected at the whole brain level. Differences related to Response to Radiation, for example, were not detected, nor were differences in the neuropeptide genes *neuropeptide F*, *tachykinin*, *sulfakinin* and *orkokinin*. Furthermore, differences in energy metabolism were not directionally concordant between the *PI* and whole brain data sets; genes related to oxidative phosphorylation are downregulated in forager whole brains but upregulated in the *PI*. Overall, these results suggest that the *PI* shows a distinct transcriptional profile that is in line with its function as a neurosecretory region of the brain. Higher expression of energy metabolism in the *PI* of foragers, for example, fits with the concomitant upregulation of insulin signaling in foragers (Ament et al., 2008), and with my results showing that *insulin-like peptide 1* is differentially expressed in the *PI* of nurse and forager bees.

In insects, neurosecretory cells in *PI* are insulin-producing cells in the brain (Wu and Brown, 2006). Insulin-signaling via these cells has been shown to have co-regulatory interactions with JH (Tatar et al., 2001). In honey bees, insulin-signaling has been shown to regulate behavioral maturation (Ament et al., 2008) but whether insulin production in the *PI* contributes to this process is not known. In my analyses, I found *insulin-like peptide 1* to be upregulated in the *PI* in forager bees. This is consistent with what has been shown in whole brain analyses. However, contrary to previous whole brain results, I did not find an upregulation of insulin signaling genes

in the *PI* due to dietary manipulations or JHA treatment (Ament et al., 2008; Corona et al., 2007). Further examination of my RNA-sequencing results showed *insulin-like peptide 1* was expressed at very low levels in these data sets. At present, I cannot determine whether this is due to a technical artifact or whether this result is biologically relevant. Future studies should investigate the expression pattern of *insulin-like peptides* in the brain and specifically test whether insulin signaling in the *PI* has a co-regulatory relationship with JH that elicits physiological and behavioral changes.

Dietary manipulations resulted in expression differences in the *PI* that were not consistent with behavioral maturation, contrary to my first hypothesis. This result is surprising given the *PI* is known to integrate nutritional changes in other insects (Rajan and Perrimon, 2012) and because the switch from a high protein diet to a low protein diet occurs naturally prior to behavioral maturation (Crailsheim et al., 1992). A possible explanation for my result is that bees are buffered from nutritional changes by high abdominal lipid levels. The nurse-age focal group used in my experiments presumably had high levels of abdominal lipids, which could provide a buffer against pollen deprivation. However, previous studies have also reported subtle effects due to dietary changes, this time for even younger bees with low levels of stored nutrients. In field trials Toth et al. (2005) showed that pollen deprivation caused a moderate reduction in lipid profiles and did not significantly induce early foraging. Similarly, Wheeler et al. (2013) showed dietary manipulations elicit relatively few changes in brain gene expression in very young bees and most of these changes were not consistent with behavioral maturation. Thus, the explanation that high levels of abdominal lipid buffer bees from the effects of dietary manipulations is not satisfying because younger bees, with lower levels of abdominal lipids, also show only modest effects of

such manipulations. These results instead suggest that honey bees have evolved an attenuation of the tradition relationship between diet and nutritional physiology, an idea I develop more completely below.

Contrary to the results for diet, I did find JHA treatment elicited expression patterns in the *PI* consistent with behavioral maturation, supporting my second hypothesis. Further, *cis* motif analyses showed maturation and JHA-induced transcriptomic changes were attributed to proposed and experimentally demonstrated, respectively, regulators of behavioral maturation such as NFkβ and *usp* (Ament et al., 2012b; Chandrasekaran et al., 2011), suggesting the *PI* may mediate worker division of labor through the action of these transcription factors. In addition, I found the transcription factor *crc* was differentially expressed between nurses and foragers and showed *cis* motif enrichment for genes upregulated by JHA treatment. This transcription factor has been shown to modulate neuropeptide signaling during insect development (Hewes et al., 2000) and may therefore perform a similar function in the *PI* within the context division of labor.

That I found thousands of genes differentially expressed in the *PI* of nurse and forager bees and hundreds in the *PI* of JHA-treated bees may reflect differences in the technologies used to generate transcriptional profiles. The *PI* nurse-forager profiles were generated using microarrays, while JHA-treated bees were profiled using RNA-seq. There are many differences between these two technologies, one of which is greater dynamic range for RNA-seq (Ozsolak and Milos, 2011). Increased dynamic range makes RNA-seq a more sensitive technology, with increased ability to detect more lowly expressed transcripts, but it may also make RNA-seq more sensitive to within group variation. The latter hinders the detection of differentially expressed transcripts

especially if samples are derived from natural populations with mid-to-high levels of genetic variation, such as those used in my experiments. These technical aspects likely had some impact on my results. However, previous analyses performed exclusively on microarrays showed similar differences in the magnitude of gene expression differences between behavioral collections and laboratory-based manipulations (Whitfield et al., 2006). Thus, my results may also reflect the distinct environments in which each of my groups was sampled. Nurse and forager bees were collected from their natural environment. Therefore, genes differentially expressed between these two behavioral groups may represent a combination of acute responses to the environment as well as long-term transcriptional differences important in keeping a bee in a specific behavioral and physiological state. In contrast, bees treated with JHA were kept under laboratory conditions and were not allowed to forage; thus, the JHA transcriptional changes may represent to the long-term, regulatory transcriptional differences between nurses and foragers. *cis* motif enrichment analyses that identify known regulators of behavioral maturation (see above) as enriched in both experiments provide some evidence for these inferences.

Based on my results and those from the literature, I propose that adult workers have evolved an attenuation of the traditional relationship between diet and nutritional physiology, and instead have evolved a relationship between social factors and nutritional physiology, mediated by JH (Figure 3.3). In support of these ideas, I found the *PI* interacts with JH in a manner consistent with behavioral maturation, and found that diet had much more subtle effects on *PI* gene expression that were not consistent with behavioral maturation. As stated above, previously reported effects of diet on worker physiology and behavior (Toth et al., 2005; M. M. Wheeler et al., 2013) are weaker than expected given that nutrition is the main environmental factor driving

JH-mediated physiological changes in most insects (D. Wheeler, 1996), and that there are major changes in worker diet during behavioral maturation (Crailsheim et al., 1992). The strongest effects linking nutrition to foraging onset have come from starvation experiments (Schulz et al., 1998; Toth et al., 2005). These studies have shown that a depletion of a colony's food stores induces early foraging; however, depriving colonies of pollen does not elicit these same effects (Toth et al., 2005). These results indicate that more moderate diet changes are not causal to foraging onset. Recent transcriptomic studies on the brain and peripheral tissues are consistent with my hypothesis by showing that diet did not elicit gene expression changes consistent with behavioral maturation (Ament et al., 2011; M. M. Wheeler et al., 2013).

Nevertheless, dramatic changes in nutritional physiology do occur during behavioral maturation and direct manipulation of key physiological factors do regulate the onset of foraging. For example, an experimental depletion of a bee's abdominal lipid levels (Toth et al., 2005) or RNAi knockdown of the lipid storage protein, vitellogenin, leads to early foraging. There is growing evidence that these physiological factors are modulated by social signals. Queen mandibular pheromone (QMP), for example, modulates the expression of the *vitellogenin* gene (Fischer and Grozinger, 2008). Similar effects on vitellogenin have been reported for brood pheromone (BP)(Smedal et al., 2009). Moreover, recent transcriptomic studies show that unlike diet, both QMP and BP affect brain gene expression in a manner consistent with behavioral maturation. Together these findings suggest that pheromones are the primary extrinsic regulators of behavioral maturation. The connection between pheromones to nutritional physiology is likely JH-mediated because QMP has been shown to modulate JH levels (Pankiw et al., 1998) and because JH has co-regulatory interactions with vitellogenin (Guidugli et al., 2005; Pinto et al.,

2000). One additional possibility is that social factors mediate nutritional physiology through interactions with *corpora allata*, the glands that produce JH, followed by the *PI*. My results provide indirect evidence for this idea by demonstrating that maturation-related changes are present in the *PI*, but that these maturational changes are not regulated by diet. Future work should test these connections directly, but if true it would further suggest that neuroendocrine systems in honey bees have evolved to regulate worker division of labor by becoming less "sensitive" to dietary inputs and to integrate social factors tied to colony-level needs.

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TABLES FOR CHAPTER 3:

Table 3.1. The number of differentially expressed genes due to diet and JHA manipulations.

Comparison	*P*<0.001 Treatment	Diet	Interaction
Overall effect (FDR<0.1)	**-**	**365**	**1**	**17**
Ctlr P- vs. Ctlr P+	62	16	0	9
JHA+P-vs. JHA+P+	73	11	1	10
JHA+P- vs.Ctrl P-	110	75	1	5
JHA+P+ vs. Ctrl P+	359	199	1	14
JHA+P+ vs. Ctrl P-	141	104	1	2
JHA+P- vs. Ctrl+P+	163	132	1	2

JHA^+ =JHA treatment; Ctrl = bees treated with acetone, P^+ = bees were fed a high protein diet; and P^- = diet with no protein.

Table 3.2. *cis* regulatory motifs and corresponding transcription factors common to Nurse-Forager and Ctrl-JHA gene lists.

Motif	Transcription Factor	Nurses	Ctrl	*Pc*
NR1H2_RXRA	NR1H2 & RXRA	0.027	0.003	0.031
tap_da_SOLEXA_5	tap & da	0.010	0.021	0.031
V_AP1_Q4	AP1	0.007	0.045	0.052
ESR2	ESR2	0.037	0.021	0.057
V_HOX13_01	HOX13	0.027	0.033	0.059
Motif	**Transcription Factor**	**Forager**	**JHA**	***Pc***
Hsf_compiled	HSF	0.005	0.005	0.010
V_NFKAPPAB50_01	NF-kappaβ	0.034	0.000	0.034
Fer1_SOLEXA_5	Fer1	0.044	0.017	0.060
gsb_SOLEXA	gsb	0.034	0.028	0.060
Nkx2-5	Nkx2	0.044	0.020	0.063
V_CAAT_C	CAAT	0.044	0.028	0.070

Numbers are *P*-values (Fisher's exact test) that denote enrichment for genes that were upregulated in Nurses vs. Foragers and Ctrl vs. JHA and *vice versa.* Significance for associations between experiments, in the expected direction, is denoted by a combined *P* value, *Pc.*

FIGURES FOR CHAPTER 3:

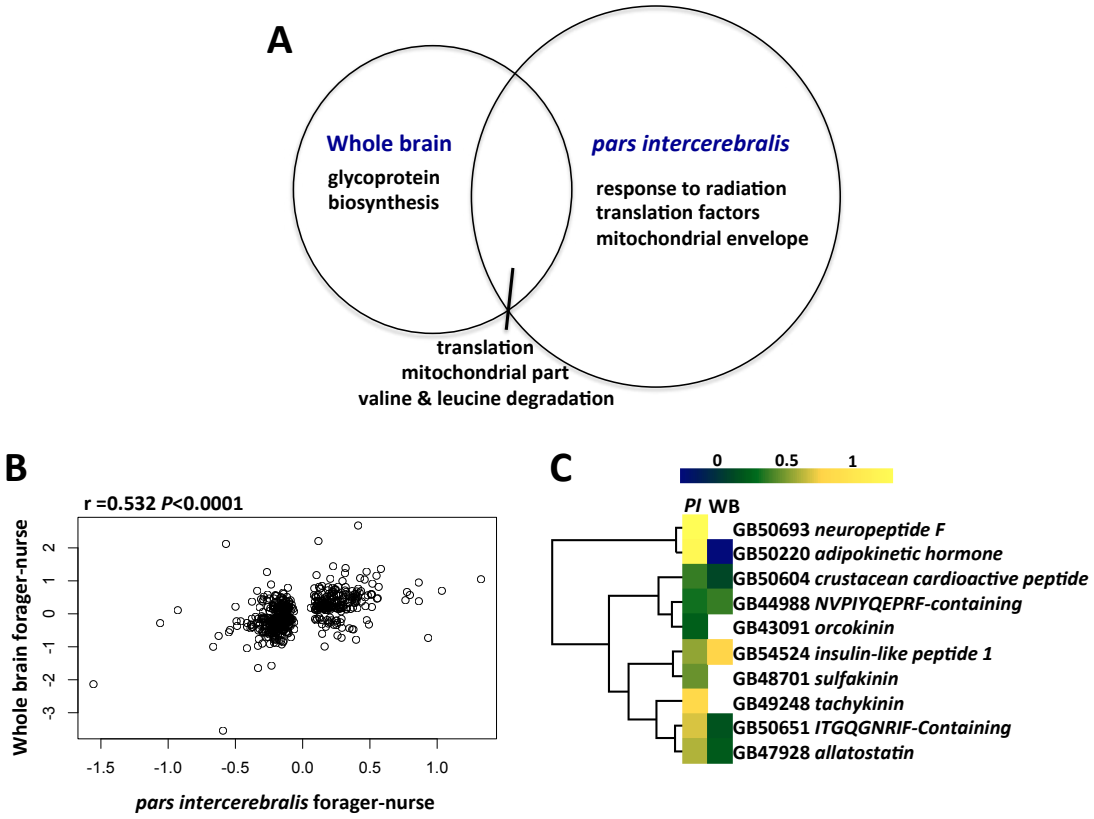

Figure 3.1: Comparison of results from nurse vs. forager *pars intercerebralis* (*PI*) and previously published whole brain (Alaux et al., 2009a) microarray experiments. (A) Venn diagram showing significant Gene Ontology terms (Fisher's exact test, $P \leq 0.001$) enriched from the list of overlapping genes (589 genes) and non-overlapping genes (*PI:* 2074 genes). (B) Pearson correlation coefficients for log-fold changes of genes that overlap between whole brain and *PI*. (C) Heat map for differentially expressed neuropeptide genes in whole brain (WB) and *PI*. Yellow indicates neuropeptide genes that are significantly upregulated in foragers, blue upregulated in nurses.

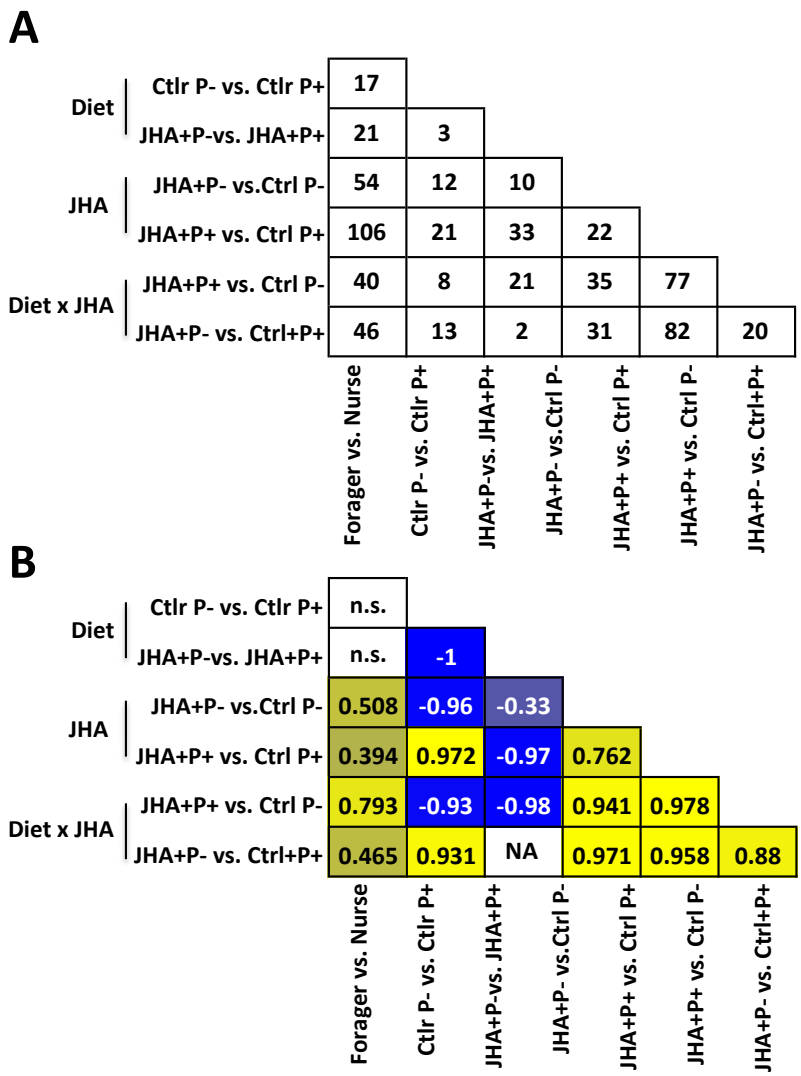

Figure 3.2: Overlap and correlation analyses for genes differentially expressed in the *pars intercerebralis* of nurses and foragers and as a function of diet and JH analog (JHA) treatments. (A) Number of genes overlapping in various pairwise comparisons. (B) Pearson's correlation coefficients for genes in Panel A. Pairwise correlations were assessed based on the predicted effect of each manipulation of foraging onset. Numbers represent the correlation coefficients that were significant (P<0.05). Yellow represents a positive correlation and blue

represents a negative correlation. JHA+ = bees treated with JHA; Ctrl = bees treated with the solvent acetone; P+ = bees fed a high protein diet and P- = bees that received a low protein diet

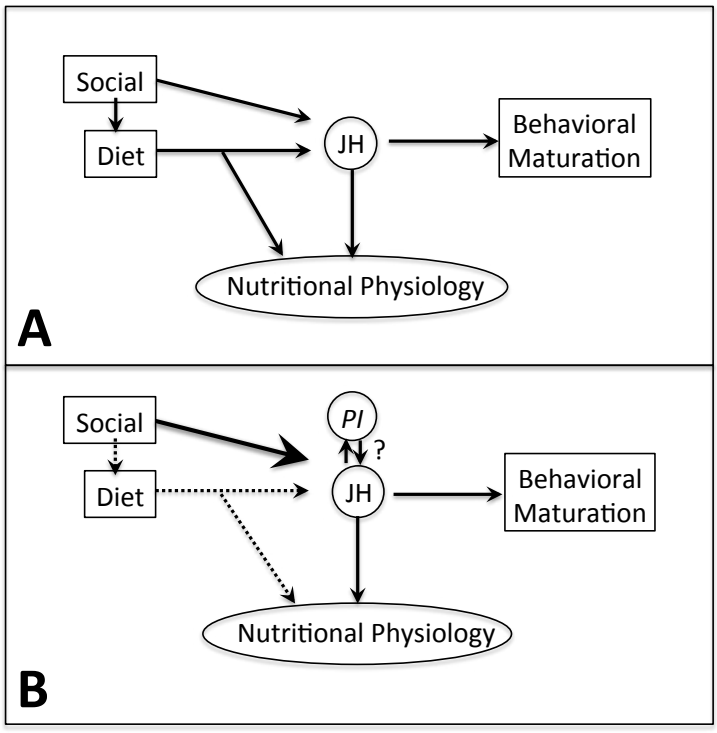

Figure 3.3: Verbal model proposing diet has weak effects on JH and nutritional physiology, and instead social factors have evolved to regulate JH and nutritional physiology. (A) Depicts previous understanding of the relationships between diet and nutritional physiology and between social factors and nutritional physiology (modified from Ament et al. 2011). (B) Represents the proposed model based on present and previous evidence. The word Social represents pheromonal cues; Diet represents pollen or sugar diets; JH = juvenile hormone; *PI* = *pars intercerebralis*; Nutritional Physiology includes abdominal lipids and vitellogenin. In Panel B, dotted lines represent an attenuation of diet signal to contrast with the stronger effects of social factors. The (?) in Panel B suggests genes in the *PI* could influence JH but this needs validation.

SUPPLEMENTARY MATERIALS FOR CHAPTER 3:

Table 3.S1. Gene Ontology enrichment analyses for genes (FDR<0.1) upregulated in the *pars intercerebralis* of foragers and nurses.

Category	Term	Count	P-value	Benjamini
Up-regulated in foragers				
GOTERM_BP_FAT	GO:0009583~detection of light stimulus	25	1.18E-11	2.48E-08
GOTERM_BP_FAT	GO:0006091~generation of precursor metabolites and energy	44	1.74E-08	5.20E-06
KEGG_PATHWAY	dme00190:Oxidative phosphorylation	32	7.97E-07	7.89E-05
GOTERM_BP_FAT	GO:0016310~phosphorylation	73	1.36E-05	0.001
GOTERM_BP_FAT	GO:0006096~glycolysis	8	0.003	0.185
GOTERM_BP_FAT	GO:0045471~response to ethanol	7	0.007	0.328
Up-regulated in nurses				
KEGG_PATHWAY	dme03010:Ribosome	47	3.30E-23	2.90E-21
GOTERM_BP_FAT	GO:0006412~translation	92	1.91E-22	3.25E-19
GOTERM_MF_FAT	GO:0008135~translation factor activity, nucleic acid binding	24	2.02E-06	4.86E-04
GOTERM_BP_FAT	GO:0006732~coenzyme metabolic process	19	0.001	0.276

P-values are a modified Fisher Exact test calculated using the number of *Drosophila* orthologs to honey bee genes as a background. Benjamini are corrected *P values* for multiple hypothesis testing.

Table 3.S2. Enriched Gene Ontology (GO) terms for genes that overlap between nurse vs. forager *pars intercerebralis* (*PI*) and whole brain datasets and GO terms enriched for genes that are only differentially expressed in *PI* relative to whole brain (*PI* specific) and *vice versa* (whole brain specific).

Category	Term	Count	P-value	Benjamini
Overlap between PI and whole brain				
KEGG_PATHWAY	dme03010:Ribosome	28	6.27E-12	5.02E-10
GOTERM_BP_FAT	GO:0006412~translation	40	1.83E-07	7.73E-05
GOTERM_CC_FAT	GO:0044429~mitochondrial part	32	0.007	0.130
KEGG_PATHWAY	dme00280:Valine, leucine and isoleucine degradation	9	0.003	0.120
PI specific				
GOTERM_BP_FAT	GO:0009314~response to radiation	32	1.91E-05	0.043
GOTERM_MF_FAT	GO:0008135~translation factor activity, nucleic acid binding	28	2.50E-04	0.225
GOTERM_CC_FAT	GO:0005740~mitochondrial envelope	59	1.75E-04	0.081
GOTERM_BP_FAT	GO:0015837~amine transport	14	0.001	0.224
Whole brain specific				
GOTERM_BP_FAT	GO:0009101~glycoprotein biosynthetic process	12	3.65E-04	0.395

*P-value*s are a modified Fisher Exact test with the number of *Drosophila* orthodox to honey bee genes as a background.

Table 3.S3. Enriched Gene Ontology (GO) terms for genes differentially expressed in diet *x* treatment comparisons.

Category	Term	Count	P-value	Benjamini
Ctrl P- vs. Ctrl P+				
GOTERM_CC_FAT	GO:0019898~extrinsic to membrane	4	8.30E-04	0.033
INTERPRO	IPR017973:Cytochrome P450, C-terminal region	3	0.001	0.050
JHA+P- vs. JHA+P-				
KEGG_PATHWAY	dme04914:Progesterone-mediated oocyte maturation	3	0.023	0.373
JHA+P- vs. Ctrl P-				
KEGG_PATHWAY	dme00903:Limonene and pinene degradation	5	8.22E-05	0.002
SP_PIR_KEYWORDS	oxidoreductase	9	0.002	0.057
INTERPRO	IPR015609:Molecular chaperone, heat shock protein, Hsp40, DnaJ	3	0.011	0.253
JHA+P+ vs. Ctrl P+				
GOTERM_CC_FAT	GO:0022626~cytosolic ribosome	9	7.13E-04	0.060
GOTERM_CC_FAT	GO:0005576~extracellular region	12	0.007	0.222
JHA+P- vs. Ctrl P+				
SP_PIR_KEYWORDS	egf-like domain	5	7.16E-04	0.058

P-value estimates are a modified Fisher Exact test calculated using the number of *Drosophila* orthologs to honey bee genes as a background.

Table 3.S4. Transcription factors that are differentially expressed in the *pars intercerebralis* of foragers and nurses. F vs. N column corresponds to the log-fold change differences between forager and nurses.

Gene	Drosophila ID	Name	F vs. N
GB50933	FBgn0003117	pnr	-1.801
GB41714	FBgn0263072	CG43347	-0.640
GB53500			-0.515
GB52304			-0.365
GB50071	FBgn0011655	Med	-0.350
GB55635	FBgn0032202	CG18619	-0.341
GB50091	FBgn0003499	sr	-0.337
GB49927	FBgn0050420	Atf	-0.292
GB40453	FBgn0033155	Br140	-0.291
GB51395	FBgn0261064	Rbsn-5	-0.287
GB44964	FBgn0032130	CG3838	-0.275
GB48690	FBgn0003715	CG16778	-0.272
GB42329	FBgn0262975	cnc	-0.253
GB49190	FBgn0003044	Pcl	-0.245
GB49419	FBgn0037445	CG9727	-0.237
GB47037	FBgn0023546	Hr4	-0.236
GB50732	FBgn0035993	Nf	-0.231
GB55970	FBgn0005655	mus209	-0.222
GB44031	FBgn0260632	dl(NF-KB)	-0.210
GB42142	FBgn0001078	ftz-f1	-0.209

GB42177	FBgn0022740	HLH54F	-0.201
GB42382	FBgn0000018	abo	-0.184
GB44032			-0.149
GB46509	FBgn0035357	MEP	-0.138
GB44656	FBgn0000289	cg	-0.135
GB44836	FBgn0004652	fru	-0.124
GB50458	FBgn0015381	dsf	-0.121
GB50553	FBgn0039038	CG6688	-0.113
GB49869	FBgn0032904	Mtp	-0.093
GB47151	FBgn0000139	ash2	-0.083
GB41522	FBgn0027950	MBD-like	0.056
GB49843	FBgn0039411	dys	0.074
GB46211	FBgn0032979	CG1832	0.086
GB43687			0.109
GB53328	FBgn0024887	kin17	0.116
GB47820			0.127
GB48366	FBgn0000370	crc	0.128
GB41753			0.128
GB41654	FBgn0013799	Deaf1	0.135
GB54092	FBgn0010825	Gug	0.139
GB55012	FBgn0011763	Dp	0.142
GB54796	FBgn0025334	PHDP	0.153
GB55837	FBgn0032940	Mio	0.156
GB55033	FBgn0004595	pros	0.158

GB54841	FBgn0003345	sd	0.159
GB46747			0.164
GB53417	FBgn0029711	Usf	0.169
GB41982	FBgn0003334	Scm	0.170
GB48171	FBgn0262732	mbf1	0.176
GB44791	FBgn0002914	Myb	0.177
GB47329	FBgn0000057	adp	0.179
GB52687	FBgn0032223	GATAd	0.189
GB44585	FBgn0021872	Xbp1	0.190
GB52039	FBgn0259938	cwo	0.203
GB41225	FBgn0033635	CG7777	0.204
GB45157	FBgn0013753	Bgb	0.210
GB44351	FBgn0036179	CG7368	0.218
GB51904	FBgn0000611	exd	0.218
GB40859	FBgn0034240	MESR4	0.223
GB54118	FBgn0259172	rn	0.226
GB52323	FBgn0037555	Ada2b	0.231
GB53164			0.240
GB43591	FBgn0024184	unc-4	0.247
GB52047	FBgn0259789	vfl	0.250
GB56017			0.251
GB54432			0.275
GB42143	FBgn0004401	Pep	0.285
GB43953	FBgn0023094	cyc	0.286

GB52426	FBgn0031759	lid	0.306
GB48239			0.311
GB46492	FBgn0014467	CrebB	0.337
GB50048	FBgn0013948	Eip93F	0.348
GB46523	FBgn0035769	CTCF	0.360
GB43847	FBgn0038851	dmrt93B	0.418
GB50795	FBgn0261953	AP-2	0.482
GB47788	FBgn0261588	pdm3	0.490
GB53167	FBgn0085432	pan	0.518
GB40407	FBgn0038402	Fer2	0.565
GB45040	FBgn0043364	cbt	0.569
GB53318	FBgn0001291	Jra	0.573
GB50651	FBgn0033358	CG8216	0.713
GB45501			0.828

Table 3.S5. **Differentially expressed transcription factors in the *pars intercerebralis* in JHA-based comparisons. Numbers are log-fold change differences between treatment groups.**

Gene	*Drosophila* ID	Name	JHA+P- vs. Ctrl+P-	JHA+P- vs. Ctrl+P+	JHA+P+ vs. Ctrl P+	JHA+P+ vs. Ctrl P-
GB47799			0.581014391	-	-	-
GB55635	FBgn0032202	CG18619	-0.605200551	-0.613162388	-0.579193321	-0.571231484
GB45427	FBgn0028420	Kr-h1	-	0.771064808	0.813462551	-
GB51060	FBgn0035625	Blimp-1	-	-0.656846691	-	-
GB44291	FBgn0263352	Unr	-	-	-	-
GB50553	FBgn0039038	CG6688	-	-	-	-0.256121323
GB49067	FBgn0033782	sug	-	-	-	-0.531342326

JHA^+ =JHA treatment; Ctrl = bees treated with acetone, P^+ = bees were fed a high protein diet; and P^- = diet with no protein.

Table 3.S6. Significant *cis* regulatory motifs for genes upregulated *PI* of nurse bees compared to foragers

Motif	Transcription Factors	*P* value	FDR
V_HAND1E47_01	HAND1/E47	0.0004	0.0581
V_AHRARNT_01	AHR & ARNT	0.0004	0.0581
V_ZID_01	ZID	0.0010	0.0952
TP53	TP53	0.0002	0.0320
TAL1_TCF3	TAL1 & TCF3	0.0010	0.0629
brk_FlyReg	brk	0.0006	0.0693
Med_FlyReg	Med	0.0006	0.0693
esg_F3_5_SOLEXA	esg	0.0015	0.0829
usp_SOLEXA	usp	0.0015	0.0829

Table 3.S7. Significant *cis* regulatory motifs for genes upregulated *PI* of bees treated with JHA+P- compared Ctrl P-

Motif	Transcription Factor	*P* value	FDR
V_NFKAPPAB50_01	NF_kappaβ	0.0003	0.0904
Dif_Rel_SELEX	dif_rel	0.0001	0.0143
lola_PW_SOLEXA	lola	0.0013	0.0980
shn_F1_2_SOLEXA_2.5	shn	0.0013	0.0980

Table 3.S8. Differentially expressed transcription factors in the *pars intercerebralis* that also showed significant *cis* motif enrichment (*P*<0.05). Numbers are log-fold change differences between treatment groups.

Gene	Drosophila ID	Name	F vs. N
cis motifs upregulated in nurses			
GB44836	FBgn0004652	fru	-0.123910025
GB50071	FBgn0011655	Med	-0.349686082
GB41654	FBgn0013799	Deaf1	0.134884931
GB42177	FBgn0022740	HLH54F	-0.200657933
GB40407	FBgn0038402	Fer2	0.565018704
cis motifs upregulated in foragers			
GB50091	FBgn0003499	sr	-0.337355623
GB53417	FBgn0029711	Usf	0.169393733
GB49927	FBgn0050420	Atf	-0.291984702
GB44031	FBgn0260632	dl	-0.209946298

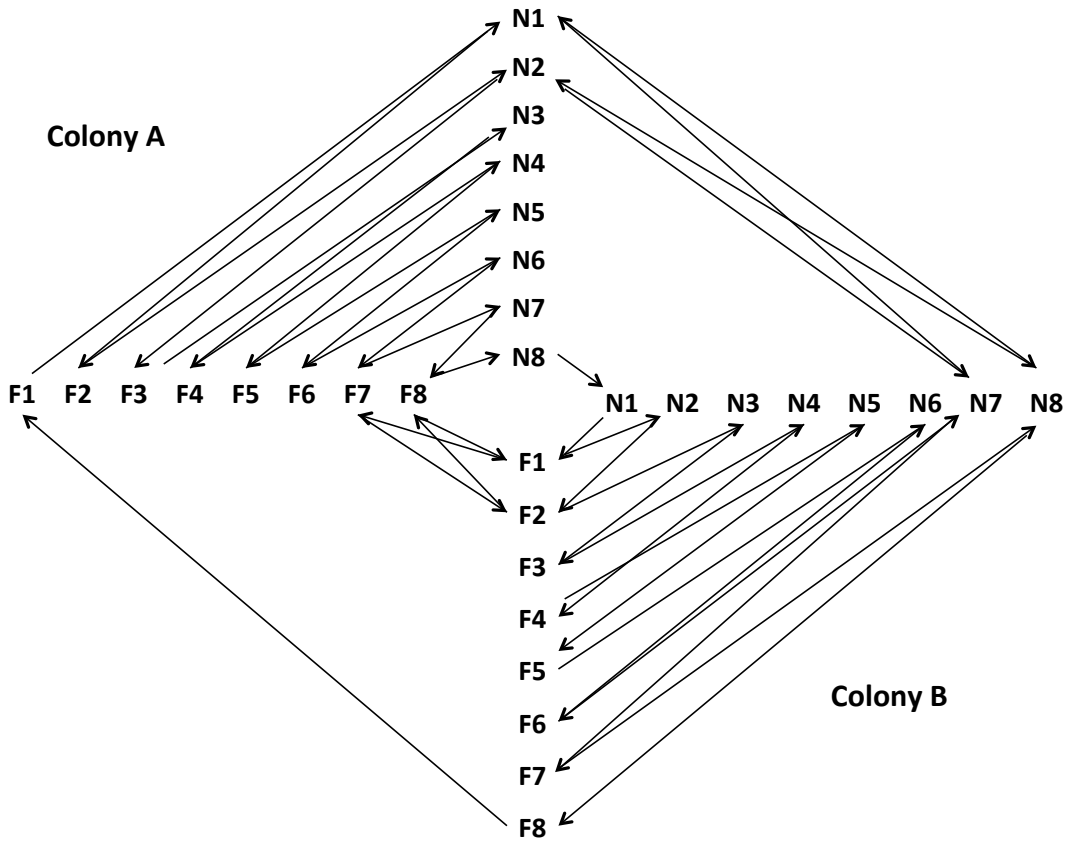

Figure 3.S1: Microarray scheme used for gene expression profiling of the *pars intercerebralis* for nurse (N) and forager (F) bees. Each arrow represents a microarray with samples at the tail end labeled with Cy3 and samples at the arrow end labeled with Cy5. This experiment included 40 microarrays to profile 32 samples, 8 samples per group, per colony.

CHAPTER 4:
DIET-DEPENDENT GENE EXPRESSION IN HONEY BEES: HONEY VS SUCROSE OR HIGH FRUCTOSE CORN SYRUP[1]

ABSTRACT

Severe declines in honey bee populations have made it imperative to understand key factors impacting honey bee health. Of major concern is nutrition, as malnutrition in honey bees is associated with immune system impairment and increased pesticide susceptibility. Beekeepers often feed high fructose corn syrup (HFCS) or sucrose after harvesting honey or during periods of nectar dearth. We report that, relative to honey, chronic feeding of either of these two alternative carbohydrate sources elicited hundreds of differences in gene expression in the fat body, a peripheral nutrient-sensing tissue analogous to vertebrate liver and adipose tissues. These expression differences included genes involved in protein metabolism and oxidation-reduction, including some involved in tyrosine and phenylalanine metabolism. Differences between HFCS and sucrose diets were much more subtle and included a few genes involved in carbohydrate and lipid metabolism. Our results suggest that bees receive nutritional components from honey that are not provided by alternative food sources widely used in apiculture.

INTRODUCTION

Honey bees are vital members of natural and agricultural ecosystems worldwide. In the United States, the Western honey bee (*Apis mellifera*) contributes more than 15 billion dollars to the

[1] Formatted for submission to Scientific Reports. Co-Author is Gene E. Robinson.

agricultural industry annually[1]. It is therefore of serious concern that honey bee populations have declined steadily in the United States, with dramatic losses of colonies starting in 2006 associated with colony collapse disorder (CCD)[2-4]. These losses have intensified the need to understand factors that impact honey bee health.

Central to honey bee health is nutrition[5]. Malnutrition in honey bee colonies can result from maintaining densities of colonies that are too high for available flora or placement of colonies for pollination of crops that are deficient in pollen or nectar or have low nutritive value. Poor nutrition can make bees more susceptible to pesticides[6] and lead to a compromised immune system making bees more vulnerable to diseases[7].

The principal natural carbohydrate source of honey bees is nectar, which is collected from flowers, transported to the hive and converted to honey for storage. This conversion involves reducing the water content to 16-20% and adding glandular secretions that contain microorganisms and enzymes, including amylases, glucose oxidases and invertases. These increase acidity and convert the sucrose in nectar into glucose and fructose[8]. The final constituents of honey vary depending on the nectar source but are mainly fructose (30-45%), glucose (24-40%) and sucrose (0.1-4.8%), as well as trace amounts of other disaccharides, vitamins, minerals, amino acids and a variety phenolic compounds[9].

Adult honey bees use honey as fuel for energy-intensive flights and colony thermoregulation[8]. Unlike larvae, adults have low levels of abdominal lipids and cannot survive for long periods of time without a carbohydrate source. A continuous supply of sugar is particularly important for

older adult bees, which specialize on foraging because they have a diet that is mainly carbohydrate-based[10]. Compared to younger bees that specialize on performing tasks inside the hive, foragers also have a higher metabolic rate [11] and lose over half their abdominal lipids stores prior to starting to forage[12].

Beekeepers often provide supplemental carbohydrates in the form of high fructose corn syrup (HFCS) or sucrose following the harvesting of honey or during periods of nectar dearth. Supplementing with HFCS became a widespread practice following early studies that showed acceptable honey bee survival[13] and equivalent honey production and long-term productivity relative to honey feeding[14]. In addition, HFCS has a fructose-to-glucose ratio similar to honey, with the most common bee feed formulation composed of 55% fructose and 42% glucose[15]. HFCS is also less expensive than sucrose and is less labor-intensive to administer as food because it comes in liquid form[16].

However, questions regarding the suitability of HFCS for honey bees have arisen, in part because of CCD and because of research showing HFCS may have deleterious metabolic effects in mammals[17]. Honey produced exclusively from HFCS had higher levels of the disaccharide, fructosyl-fructose[18] and caused decreased spring brood and wax production relative to sucrose[19]. Moreover, there is growing evidence that constituents in honey, absent from sucrose and HFCS, positively affect the honey bee's xenobiotic detoxification system[20,21]. These results suggest that honey, sucrose and HFCS may impact honey bee physiology and health differently.

We explored this issue further with whole-genome transcriptomics to comprehensively survey

the effects of honey, sucrose and HFCS on fat body gene expression. The fat body is a multifunctional organ responsible for nutrient storage, energy mobilization and the production of antimicrobial peptides. Nutrient storage and mobilization are coupled to hormonal signals that include insulin and adipokinetic hormone to fulfill ongoing physiological demands[22]. In adult honey bees, the fat body is known to be transcriptionally responsive to nutritional manipulations and manipulations that affect aging and health[23,24]. We focused on the fat body to study the effects of different dietary carbohydrate sources on the expression of genes involved in hormonal signaling, nutrient storage, energy metabolism, and immune function. In this study, we used older bees because their diet is primarily carbohydrate-based[10] and because older bees have been shown to be the primary consumers of carbohydrate supplements inside the hive[5].

RESULTS

Measurements taken daily throughout the week-long trials showed similar levels of food consumption for bees fed honey, HFCS or sucrose (0.040±0.001, 0.036±0.002, 0.032±0.003g/bee/day, respectively, F=4.26 *P*=0.055). Mortality also did not vary between diets (F=0.57 *P*=0.59) and was between 0-7 % across all cages.

RNA-sequencing (RNA-seq) was performed to examine the effect of each diet treatment on fat body gene expression. Initial examination of our results revealed that one of our colony replicates was heavily infected with deformed wing virus (DWV). For Colony A, an average of 37.06±8.66% reads aligned to the DWV genome sequence compared to only 1.29±2.77% for Colony B. By contrast, an average of 53.81±0.08% of the RNA-seq reads mapped to the honey

bee genome for Colony A, while for Colony B the average was 87.72±0.03%. Due to this difference, we analyzed each colony separately. We explored the effects of diet treatment using multidimensional scaling (MDS) plots (using the log fold change of the top 100 genes) generated for each colony. These analyses indicated that chronic feeding of sucrose and HFCS elicited gene expression profiles distinct from bees fed a honey diet (Table 4.1A and 4.1B). This pattern was observed for both colonies, indicating that the different responses to diet treatments occurred regardless of differences in apparent viral load.

To probe more extensively for diet effects on gene expression, we analyzed the results from both colonies together, assessing the main effect of diet treatment with colony as a blocking factor. Honey elicited hundreds of differences in gene expression relative to HFCS and sucrose (Figure 4.2). There were 104 genes differentially expressed (FDR <0.1) in bees fed honey or HFCS and 220 genes differentially expressed between bees fed honey or sucrose. By contrast, differences between HFCS and sucrose diets were much more limited with a total of 8 genes differentially expressed.

Class prediction analyses using the support vector machine algorithm[25] revealed that diet-induced fat body gene expression changes were robust and consistent across samples. Class membership was predicted correctly with 96% (Honey vs. Sucrose), 97.5% (Honey vs. HFCS) and 100% (HFCS vs. Sucrose) accuracy, corresponding to sensitivity values (true positives identified) between 0.92-1 and specificity values (true negatives identified) of 0.95-1. Top predictors for each diet comparison are shown in Figure 4.S1. Notable among top predictor genes for the honey-based comparisons were *glutathione S transferase O3* (GB44803) and *pale*

(GB40967), which are associated with xenobiotic detoxification and tyrosine metabolism, respectively. *Maltase B1* (GB54549) and two other genes involved in energy metabolism, GB50596 (GO term: oxido-reductase activity) and GB48029 (GO term: acyl carnitine transporter activity), were top predictors for the HFCS vs. Sucrose comparison.

Consistent with class prediction analyses, Gene Ontology (GO) enrichment analyses showed that the lists of genes upregulated by honey were enriched for genes involved in amino acid metabolism and oxidation reduction (Figure 4.2, Table 4.S1), especially phenylalanine and tyrosine metabolism. These included *pale, henna* (GB48022) and *homogentisate 1,2-dioxygenase* (GB53288). Relative to sucrose, honey also upregulated the gene *flavin monooxygenase 1* (GB42239), which was associated with oxidation reduction and alkaloid detoxification[26]. By contrast, sucrose upregulated genes that were associated with axonogenesis, anion transport, and several transcription factors associated with organ development, such as *doublesex* (GB55036), *knot* (GB42304) and *tolkin* (GB52106), while HFCS upregulated the transmembrane receptors, *domeless* (GB42244) and *tyramine receptor* (GB47385) (Figure 4.2, Table 4.S1). No GO terms were enriched for the 8 genes differentially expressed between HFCS- and sucrose-fed bees. These genes included *maltase B1* (GB54549) and a diaglyceride acyltransferase (GB54888).

To gain further insights into the biological significance of these diet-induced differences in fat body gene expression, we compared our results to three previous transcriptomic (microarray) experiments on nutritional aspects of behavioral maturation in honey bees[23]. Behavioral maturation in honey bees involves a switch from a high protein to a high carbohydrate diet and a loss of approximately 50% of fat body lipids[12] prior to the shift from working in the hive to

foraging. This behavioral and physiological shift is mediated, in part, by a juvenile hormone induced reduction in the blood titers of the storage protein vitellogenin (Vg)[27]. We compared our results to three microarray experiments: 1) Maturation (hive bees compared to foragers); 2) *vg* knockdown (*vg* RNAi compared to control); and 3) Diet [bees fed a high protein diet (45% pollen, 45% honey, 10% water) compared to sucrose (50% w/v)]. We detected significant overlap between previously reported changes in fat body gene expression that occur during the hive to foraging transition[23] and our present Honey vs. Sucrose and Honey vs. HFCS gene lists (Table 4.1). Surprisingly, the fat body gene expression profile of the nutritionally enriched hive bees was more similar to that of the sucrose-fed bees, while the profile of the more nutritionally deprived foragers was more similar to that of the honey-fed bees (Table 4.1). GO enrichment analyses showed that the subset of genes overlapping the Honey vs. Sucrose or Honey vs. HFCS diets and the maturation-related experiment were associated with protein metabolism and oxidation reduction (Table 4.S2). There also was a significant overlap between the Honey vs. Sucrose and Honey vs. HFCS gene lists and the gene list from the *vg* RNAi experiment[23]; however, the log fold changes for overlapping genes were not significantly correlated indicating gene lists were not directionally similar (Table 4.1). There was no significant overlap between our Honey vs. Sucrose and Honey vs. HFCS gene lists and the Diet experiment[23], but the genes that did overlap showed a significant positive correlation that suggested honey's effects were directionally concordant to those of the Pollen+Honey treatment (Table 4.1).

DISCUSSION

A honey diet elicited a transcriptional profile distinct from sucrose and HFCS diets. These differences were present in two different honey bee colonies, with vastly different viral loads, indicating the impact of honey on fat body gene expression is robust. These results suggest that constituents in honey differentially regulate physiological processes and that sucrose and HFCS may not be equivalent nutritional substitutes to honey.

Gene Ontology enrichment analyses showed honey upregulates genes associated with processes such as "aromatic amino acid family metabolic process", as well as "oxidation reduction". Among the genes in these categories were orthologs for the *Drosophila melanogaster* genes *pale* and *henna,* which are related to phenylalanine and tyrosine metabolism. These amino acids have been linked to the production of neurotransmitters[28], and in the case of *pale* to immune responses to infection[29]. Honey additionally upregulated the gene *glutathione S transferase O3,* whose activity is known to be induced by plant compounds and to have toxicological significance in the presence of pesticides[30]. HFCS and sucrose relative to honey resulted in the upregulation of different biological processes. Sucrose, for example, upregulated processes such as axonogenesis but it is unlikely that axonogenesis is upregulated in our fat body samples; rather this GO category reflects upregulation of signaling pathways that play different roles in different tissues. HFCS upregulated the transmembrane receptors *domeless* and *tyramine receptor* suggesting differences in JAK-STAT signaling and tyrosine signaling between HFCS and honey.

However, sucrose and HFCS elicited a remarkably similar fat body transcriptional response. This result is consistent with previous studies showing no differences in colony productivity due to these diets[14] but contrasts with findings showing differences in wax production and honey bee survival due to sucrose or HFCS[13,19]. Our results suggest that bees may not be sensitive to increased fructose consumption because we detected little evidence that reflects the types of changes in hormonal signaling, energy metabolism and nutrient storage associated with high fructose corn syrup and increased fructose consumption in mammals [17 31]. Future research should test whether greater differences in gene expression due to sucrose or HFCS feeding are observed in other tissues in honey bees or whether the few differences observed in this experiment can account for differences in colony performance in more natural conditions.

To understand whether the gene expression differences associated with a honey diet relate to maturation-related physiological changes, we compared our results with previous fat body microarray studies[23]. These comparisons showed significant enrichment between honey-based gene lists and genes differentially expressed between hive bees vs. foragers as well as with gene expression differences associated with *vitellogenin* RNAi treatment. Functional analyses suggest that shared changes in gene expression were related to protein metabolism and oxidation reduction, suggesting these processes are responsive to direct diet manipulations and maturational changes. Contrary to our expectations, we found the transcriptional profile of honey-fed-bees resembled foragers rather than nurse bees. This indicates Honey vs. Sugar does not closely resemble the fat vs. skinny state characteristic of nurses and foragers but rather that there may be compounds in honey that modulate honey bee physiology towards a forager-like state.

We did not find significant enrichment between Honey vs. Sugar gene lists and bees that received a diet of Pollen+Honey or sucrose. This result is surprising because the Honey vs. Sucrose contrast is embedded within this diet-related microarray experiment. This indicates that pollen is largely responsible for the gene expression changes in the Diet microarray experiment and that those changes are separate from those elicited by honey in our experiment. Lack of statistical enrichment with pollen-induced changes partially reflects the relatively low level of protein in honey. In addition, there were differences in the age of the bees assayed in each experiment: the diet-related microarray investigated the effect of diet treatment on younger bees able to digest pollen, while our experiment assayed older bees with a decreased capability to digest pollen. Thus, the transcriptional differences elicited by a honey diet cannot be directly attributed to pollen traces in honey.

Our goal was to perform a broad unbiased survey for the effects of honey, sucrose and HFCS on honey bee physiology. Our result that honey – but not sucrose or HFCS – upregulates genes associated with protein metabolism and oxidation reduction is indicative that honey elicits health-related physiological differences. We performed our experiment using older bees that typically consume sugar solutions inside the hive and do not digest pollen; therefore, constituents in honey may provide critical nutritional components and inducers that are otherwise absent in this age group. Previous research has already identified honey constituents that upregulate detoxification pathways in the gut[21]; our results further show honey induces gene expression changes on a more global scale. These changes may have toxicological relevance under natural

conditions in contemporary agroecosystems, where bees are routinely exposed to toxins and pesticides.

METHODS

Bees

We used bees from honey bee colonies from the University of Illinois Bee Research Facility, Urbana, IL, maintained according to standard beekeeping practices. The bees were a mixture of European subspecies typical of this region. To minimize genetic variation within a replicate, we used adult worker bees from a colony derived from a queen inseminated by single male; due to haplodiploidy, these bees were related to each by an average coefficient of relatedness of 0.75. The experiment was replicated in two independent trials, each time using bees from different, unrelated, colony.

Feeding Trials

We used adult bees between the ages of 18-21 days old. These are older bees that readily consume various carbohydrate sources in the hive[5]. To obtain focal bees we removed honeycomb frames containing pupae, placed them in an incubator (34˚C/ 30% RH), marked newly emerged one-day-old bees with a spot of paint (Testor's Paint, Rockford, IL, USA) on the dorsal surface of the thorax and reintroduced the marked bees into their natal colony; this was repeated three consecutive days to obtain a base population of >500 marked bees in the hive. Focal bees were

collected when they were 18-21 days old, placed into Plexiglas cages (10x10x7 cm; 15 bees per cage) and assigned a diet treatment in the laboratory. Diet treatments consisted of 50% (w/v) honey, 50% high fructose corn syrup (HFCS 55) or 50% sucrose *ad libitum* for each cage replicate (N = 3 cage replicates per treatment). All cages were kept in a 29°C incubator. Consumption and mortality were monitored daily for 7 days. After this time period, bees were flash-frozen and stored at -80°C for analysis.

Dissections and RNA extractions

Dissections were performed by incubating an abdomen in chilled RNA-later ICE (Ambion) at -20°C for a minimum of 16 h. The gut and ventral tissue were then removed and RNA extraction performed on the fat body and adhering dorsal cuticle (RNeasy kit, Qiagen, with a DNAse treatment).

cDNA library construction and RNA-sequencing

We constructed cDNA libraries using pooled total RNA from fat body tissue from 3 individual bees. Pooling was performed to minimize sample variability within treatment groups. Each cDNA library was prepared with 1.5 µg of pooled total RNA and constructed using the NEXTflex™ Directional RNA-seq kit (Bioo Scientific) with an added mRNA purification step using Dynabeads® Oligo(dt)^{25} (Invitrogen). Library concentrations were quantified using Qubit® fluorometric quantitation and by quantitative real-time PCR using Kapa Library Quant kits (KapaBiosystems). Average fragment size and overall quality was evaluated with the

Agilent 2100 Bioanalyzer platform and an Agilent High Sensitivity DNA kit. For sequencing, all libraries were diluted to a 6nM concentration. In total, we sequenced 5 libraries per treatment per colony, for a total of 30 libraries. Ten libraries were sequenced per lane (2-4 libraries per diet treatment/per lane) with an Illumina HiSeq2000 instrument and were sequenced as single-end, 100 nt reads.

Bioinformatics Pipeline

RNA sequencing generated an average of 19,200,920 reads per library. Reads from each library were aligned against the *Apis mellifera* genome, Assembly 4.5[32] using Tophat [33]and read counts were generated for genes using HTSeq v0.5pv2 (http://www-huber.embl.de/users/anders/HTSeq) and the *Apis mellifera* Official Gene Set, version 3.2. Reads that did not map uniquely or that mapped to genomic locations outside genes were not included in analyses for differential expression. We identified differentially expressed genes (DEGs) using a generalized linear model in the EdgeR package, version 3.2.4[34].

Because the number of differentially expressed genes in this study was small, we performed an additional analysis to test the robustness of the results. We compared differences in fat body gene expression between two subsets of the entire group of individuals fed on honey (n=5 per subset), permuting the samples allocated to each subset a total of 100 times. We found only a few genes (2.07±0.344 genes (FDR<0.1)) to be differentially expressed in these comparisons, suggesting that the differences we report between diet treatments are reliable.

Identification of Deformed Wing Virus Infection

Reads that did not align to the *Apis mellifera* genome were queried using BLAST on the NCBI website. BLAST results identified unaligned reads from Colony A as deformed wing virus (DWV) sequences. We further validated DWV infection by aligning all samples to the DWV genome (ftp.ncbi.nlm.nih.gov/genomes/Viruses/Deformed_wing_virus_uid14891), using Bowtie 2.

Class Prediction Analyses

To identify the most robust and consistent changes in fat body gene expression caused by each diet, we performed class prediction analyses using the support vector machine algorithm with 5-fold cross-validation. Class prediction analyses were performed using CMA[25], with normalized read counts for genes with a *P* value <0.05. Top predictors for each diet contrast were selected based on their ranking as a top classifier in at least 10 of the 50 iterations.

Gene Ontology Analysis

Inferences for major functional themes for each DEG list were drawn from GO enrichment analyses using the DAVID Bioinformatic Resources 6.7 functional annotation tool[35]. These analyses were performed using the *Drosophila melanogaster* orthologs associated with each

DEG list. Statistical analyses of enrichment were performed with a hypergeometric test, using the number of honey bee genes with annotated *Drosophila* orthologs as the reference.

Comparisons to Previous Microarray Studies

Ament et al. (2011) previously published microarray studies investigating fat body gene expression related to behavioral maturation. We compared our results to three microarray experiments: 1) Maturation (hive bees compared to foragers; 2) *vitellogenin* knockdown (*vitellogenin* dsRNA compared to control; and 3) Diet: [bees fed a high-protein diet (45% pollen, 45% honey, 10% water) compared to sucrose (50% w/v)].

To determine whether the lists of DEGs contained significant levels of overlap, we calculated an enrichment factor (RF) by dividing the observed number of overlapping genes by the expected number. The expected number of overlapping genes was calculated by multiplying the length of each DEG list and then dividing this value by the total number of genes included in the analyses[36]. We tested for significant enrichment using a hypergeotmetric test (1-tailed) with the p-hyper function in R. To determine whether the overlapping genes between two lists were directionally concordant, we compared the log-fold changes for each gene list and assessed significance with a Pearson's correlation.

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TABLES FOR CHAPTER 4:

Table 4.1: Enrichment and correlation analyses for honey-related contrasts and maturation-related microarray experiments. Pairwise comparisons are for genes differentially expressed at an FDR<0.1. The number of genes compared is shown next to each experiment name in parentheses. RF is an enrichment factor for the number of genes that overlapped and *P* represents significant enrichment using a hypergeometric test. Correlations results were calculated with genes that overlapped between pairwise comparisons.

Experiment	Nurse vs. Forager (2154)			Control vs. *vg* RNAi (2823)			Pollen+honey vs. sucrose (2449)		
	Overlap	RF	Correlation (r)	Overlap	RF	Correlation (r)	Overlap	RF	Correlation (r)
Honey vs. Sucrose (220)	86	1.306 (*P*=0.002)	-0.261 (*P*=0.03)	162	1.906 (*P*<0.0001)	0.051 (*n.s.*)	70	0.935 (*n.s.*)	0.662 (*P*<0.0001)
Honey vs. HFCS (104)	45	1.446 (*P*=0.002)	-0.127 (*n.s.*)	75	1.866 (*P*<0.0001)	-0.129 (*n.s.*)	40	1.130 (*n.s.*)	0.645 (*P*<0.0001)

FIGURES FOR CHAPTER 4:

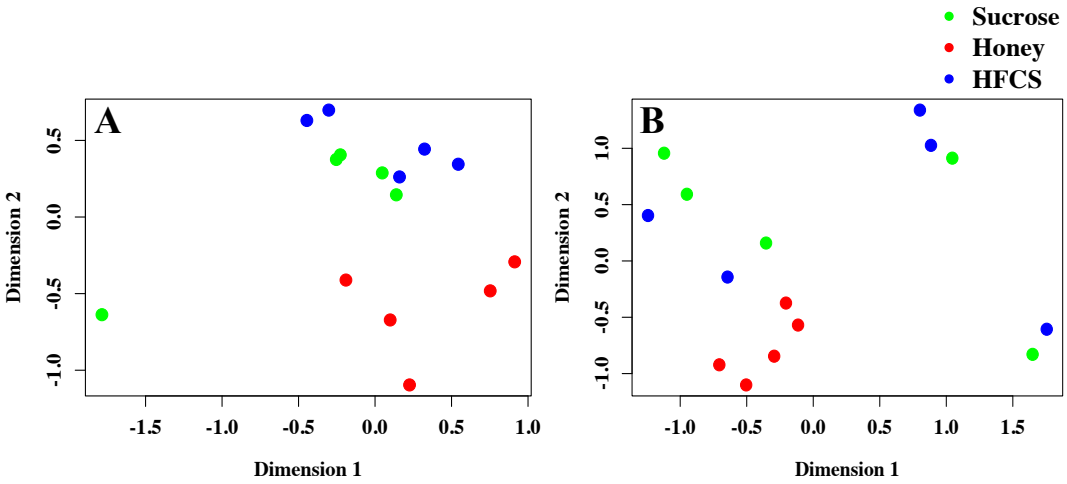

Figure 4.1: Multidimensional scaling (MDS) plots generated separately for each colony. (A) shows MDS plot for the colony heavily infected with deformed wing virus (Colony A). (B) shows MDS plot for the colony with lower levels of viral infection (Colony B). Green represents sucrose fed bees, blue represents bee fed high fructose corn syrup and red is for bees fed honey. Plots were generated using plotMDS function in edgeR, using normalized counts and log fold changes of the top 100 genes.

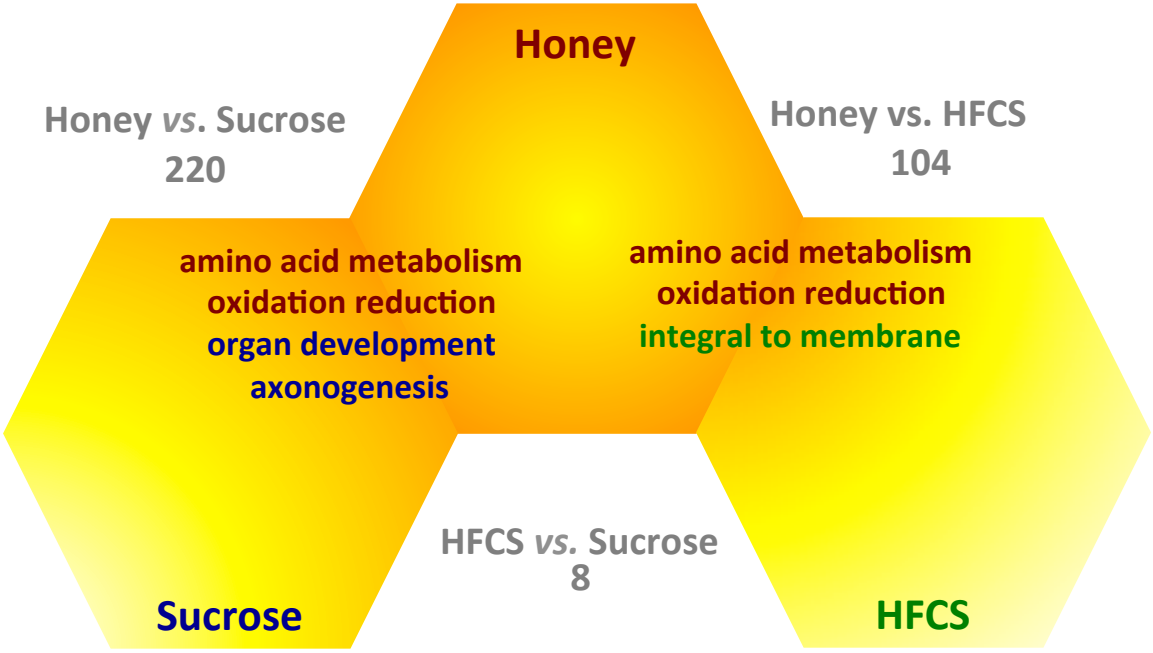

Figure 4.2: The number of significantly different genes (FDR<0.1) between diet treatments and Gene Ontology (GO) categories associated with each gene lists. The size of each gene list and appropriate diet comparison is shown in gray text. GO categories enriched ($P<0.03$) for each diet comparison are colored red if they were upregulated in response to honey, blue if they were upregulated by Sucrose relative to honey and green if they were upregulated by HFCS relative to honey.

SUPPLEMENTARY MATERIAL FOR CHAPTER 4:

Table 4.S1: Gene Ontology enrichment analyses for the honey vs. sucrose and honey vs. HFCS contrasts. Analyses were performed for genes significantly different between these two diet treatments (FDR<0.1), using honey bee orthologs to genes from *Drosophila melanogaster*.

Category	Term	Count	P-value
Upregulated by Honey relative to Sucrose			
GOTERM_BP_FAT	aromatic amino acid family metabolic process	7	5.56E-12
GOTERM_BP_FAT	L-phenylalanine metabolic process	3	3.65E-05
GOTERM_BP_FAT	biogenic amine biosynthetic process	3	0.0014
GOTERM_BP_FAT	oxidation reduction	6	0.0037
Upregulated by Sucrose relative to Honey			
GOTERM_BP_FAT	axonogenesis	7	6.31E-04
GOTERM_BP_FAT	cell projection organization	9	6.52E-04
GOTERM_BP_FAT	anion transport	4	0.0020
GOTERM_CC_FAT	intrinsic to membrane	14	0.0261
GOTERM_BP_FAT	post-embryonic organ development	6	0.0213
Upregulated by Honey relative to HFCS			
GOTERM_BP_FAT	aromatic amino acid family metabolic process	6	2.37E-11
UP_SEQ_FEATURE	metal ion-binding site:Iron	3	4.16E-05
GOTERM_BP_FAT	oxidation reduction	5	0.0017
Upregulated by HFCS relative to Honey			
GOTERM_CC_FAT	intrinsic to membrane	7	0.0027

Table 4.S2. Gene Ontology terms enriched for genes that overlapped between honey-related contrasts and maturation-related microarray experiments. Number of genes within each term is shown in each column with *P* values in parentheses. Analyses were performed using honey bee orthologs to genes from *Drosophila melanogaster*.

	GO Term	Nurse vs. Forager	Control vs. *vg* RNAi	Polllen+Honey vs. Sucrose
Honey vs. Sucrose	aromatic amino acid family metabolic process	7 (1.47E-10)	7 (3.31E-08)	7 (2.50E-10)
	L-phenylalanine metabolic process	3 (9.58E-05)	3 (5.32E-04)	3 (1.13E-04)
	extracellular region	5 (0.002)	-	-
	metal ion-binding site:Iron	3 (4.94E-04)	-	3 (6.16E-04)
Honey vs. HFCS	aromatic amino acid family metabolic process	6 (1.06E-10)	6 (4.81E-09)	6 (3.32E-10)
	Tyrosine Metabolism	5 (8.51E-07)	-	5 (8.51E-07)
	metal ion-binding site:Iron	3 (8.31E-05)	3 (3.85E-04)	-

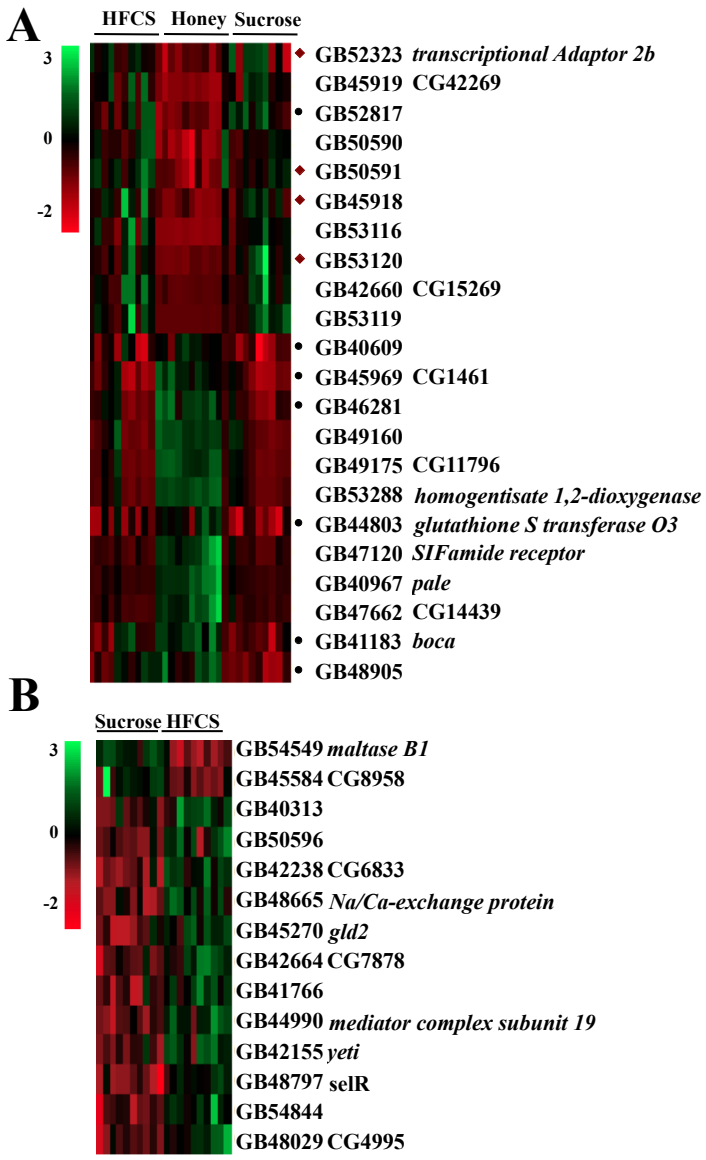

Figure 4.S1: Heat map depicting log fold changes (median-centered values) for top predictor genes of diet treatments. Columns represent samples for each specified diet treatment and rows represent genes. Honey bee accession numbers are shown for each gene as well as the gene name for the corresponding *Drosophila melanogaster* ortholog, if present. (A) shows a joint list of top predictor genes for Honey vs. Sucrose and Honey vs. HFCS contrast. (•)

denotes top predictors for the Honey vs. Sucrose contrast only. (◆) are for top predictors specific to the Honey vs. HFCS contrast. (B) show top predictors for the HFCS and sucrose comparison.